THE PLASMA MEMBRANE LIPID RAFTS/CAVEOLAE-MEDIATED PACAP SIGNALING IN PC12 CELLS

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LIST OF PUBLICATIONS:

Weishi Zhang, Wei Duan, Nam Sang Cheung, Zhili Huang, Ke Shao and Qiu-Tian Li (Aug 2007), **"Pituitary adenylate cyclase-activating polypeptide induces translocation of its G-protein-coupled receptor into caveolin-enriched membrane microdomains, leading to enhanced cyclic AMP generation and neurite outgrowth in PC12 cells". Journal of Neurochemistry doi:10.1111/j.1471-4159.2007.04813.x**

Weishi Zhang and Qiu-Tian Li, **"Caveolae-mediated activation and nuclear translocation of ERK by Pituitary adenylate cyclase-activating polypeptide is dependent on Rap1 activation and subsequent GSK3β activity in PC12 cells". Under preparation.**

K Shao , Q Hou , M Go , W Duan , N Cheung , S Feng , K Wong , A Yoram , W Zhang , Z Huang , Q Li (Feb 2007), "**Sulfatide-tenascin interaction mediates binding to the extracellular matrix and endocytic uptake of liposomes in glioma cells". Cellular and Molecular Life Sciences 64 (4), 506-515.**

ABBREVIATIONS USED IN TEXT

AC	Adenylate cyclase
Acrylamide	N, N'-methylenbisacrylamide electrophoresis prity reagent
ADP	Adenosine-5'-diphosphate
APS	ammonium persulfate
ATCC	American type culture collection
ATP	Adenosine-5'-triphosphate
BSA	Bovine serum albumin
cAMP	Adenosine 3',5'-cyclic Monophosphate , Sodium Salt
Cho- MβCD	Cholesterol-methyl--β-cyclodextrin
CO2	Carbon dioxide
CD	cyclodextrin
CREB	cAMP responsive element binding protein
CTxB-Alexa 488	Alexa Fluor®488-cholera toxin subunit B conjugate
CTxB-Alexa 594	Alexa Fluor®594-cholera toxin subunit B conjugate
CytoD	CytochalasinD
dbcAMP	Adenosine 3',5'-cyclic Monophosphate,N^6,O^2-Dibutyryl-, Sodium Salt (cAMP analog)
DMSO	Dimethyl sulfoxide
ERK1/2	Extracellular signal-regulated kinases

EGTA	Ethylene glycol bis (2-aminoethyl-ether)-N, N, N', N'-tetraacetic acid
GM1	Gangliosides GM1
GPCR	G-Protein coupled receptor
GSLs	Glycosphingolipids
Jasp	Jasplakinolide
MAPK	Mitogen-activated protein kinase
MβCD	Methyl-β-cyclodextrin
NB-DNJ	N –Butyl-deoxynojirimycin
NGF	Nerve growth factor
PAC1R	PACAP receptor type I
PACAP	Pituitary adenylate cyclase activating polypeptide
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered Saline
PFA	Paraformaldehyde
PI	Propidium iodide
PKA	Protein kinase A
PKC	Protein kinase C
PMA	Phorbol 12-Myristate 13-Acetate
PMSF	Phenylmethylsulfonyl fluoride
PVDF	Polyvinglidene fluoride
Rap1	Member of RAS oncogene family

Ras	Retrovirus-associated DNA sequences
RP-cAMP	RP-Adenosine 3', 5'-cyclic monophosphorothioate
RNAi	RNA interference
SDS	Sodium dodecyl sulfate
siRNA	Small interfering RNA
TEMED	N, N, N', N'-tetramethyl-ethylenediamine
U18666A	3β-[2-(diethylamino)ethoxy]androst-5-en-17-one
8-OM-cAMP	Adenosine3', 5'-cyclic Monophosphate, 8-(4-Chlorophenylthio) - 2'-O-Methyl-, Sodium Salt

SUMMARY

Pituitary adenylate cyclase-activating polypeptide (PACAP), a member of the secretin/glucagon/vasoactive intestinal peptide family, which is expressed throughout the nervous system, binds to the PACAP-specific G-protein-coupled receptor family members to promote both neuronal differentiation and survival. Although the PACAP receptor is known to activate its effector protein, adenylate cyclase (AC), and thus enhance cAMP generation, the molecular mechanism utilized by the receptor to activate AC is lacking. In addition, plasma membrane lipid rafts/caveolae microdomains, which are enriched in cholesterol and glycosphingolipids, mediate many intracellular signaling cascades leading to a variety of biological outcomes. The aim of this study is to elucidate the role of lipid rafts/caveolae in PACAP-induced neuritogenesis and the pertinent signaling pathways in PC12 cells.

The current study shows that PACAP induces neurite outgrowth in PC12 cells by induction of translocation of the PACAP type 1 receptor, PAC1R, into caveolin-enriched Triton X-100-insoluble microdomains, leading to stronger PAC1R-AC interaction and elevated cAMP production. Moreover, it has been demonstrated that translocation of PAC1R is blocked by various treatments that selectively disrupt caveolae and, as a result, intracellular cAMP level is decreased and consequently the PACAP-induced neurite outgrowth retarded. In contrast, addition of exogenous ganglioside GM1 to the cells shows the opposite effects. In addition, the distribution

of the downstream effector protein Rap1 which is activated upon cAMP elevation favors the detergent-insoluble fraction upon PACAP induction. Rap1 regulates the PACAP-induced neurite outgrowth through modulating the GSK3β activity and subsequent ERK1/2 phosphorylation. These results therefore identify the PACAP-induced translocation of its G-protein-coupled receptor and Rap1 into lipid rafts/caveolae, where both AC and the regulating G-proteins reside, as the key molecular events in activating AC and inducing cAMP-mediated differentiation of PC12 cells. In addition, PACAP-elicited Rap1 and Ras activation accounted for the sustained and transient activation of downstream ERK1/2 activation and neurite extension. At the same time, PKC and Ca^{2+} mobilization collaborated with cAMP pathways on PACAP signaling by regulating GSK3β and ERK1/2 activity. ERK1/2 translocated to nucleus and activated the transcription factors CREB and Elk, which is an essential transcriptional requirement for the effective cell differentiation.

These results therefore demonstrate novel lipid rafts/caveolae-mediated signaling cascades induced by PACAP in PC12 cells. Moreover, this study shows that lipid rafts/caveolae microdomains regulated the PACAP-stimulated differentiation in PC12 cells as a platform to gather related signaling molecules upon ligand-receptor binding. Specifically, it demonstrates that the PAC1 receptor and downstream Rap1 protein preferably shift to the rafts fraction once activated, which initiated and transduced the signaling cascades. It also indicates that the integrity of lipid rafts/caveolae is of importance for the efficient neuritogenesis in PC12 cells. This study should therefore

shed light on the better understanding of the underlying mechanisms of neuritogenesis process, particularly in the neurodegenerative diseases, which may lead to more effective prevention and therapy of these diseases.

CHAPTER 1. INTRODUCTION

Elucidation of the underlying molecular and cellular basis of neuronal differentiation has been greatly facilitated by cell culture models. The cell line PC12 was cloned from rat adrenal pheochromocytoma, and it was the first cell line that was characterized based on its capacity to cease proliferation and extend branching varicose processes (ie. differentiation) when exposed to nerve growth factor (NGF), the first known neurotrophin (Greene and Tischler 1976; Levi-Montalcini 1965). In addition to NGF, it has been reported that many other cellular factors such as growth factors (e.g. platelet-derived growth factor: PDGF and fibroblast growth factor: FGF), hormones and neurotransmitters could also initiate multiple signaling pathways that converge on specific cellular targets, resulting in neurite extension and competence for neuronal excitability in PC12 cells (Vaudry et al. 2002a). Based on its ability to differentiate toward sympathetic neurons after exposure to different cellular factors, PC12 cells have been extensively used as a useful *in vitro* model for the investigation of neuronal development.

1.1. PC12 cell differentiation and signaling pathways

1.1.1. NGF pathway

Over the past 30 years, the effects of NGF on PC12 cell differentiation have been extensively investigated (Lange-Carter and Johnson 1994; Angelastro et al. 2000; Huang et al. 2001; Vaudry et al. 2002a; Lee et al. 2005). NGF-stimulated PC12 cells cease division, extend neurites, express neuronal specific markers, and become electrically excitable (Tischler and Greene 1975; Greene and Tischler 1976). In PC12 cells, the multiple effects induced by NGF appear to be mediated by at least two distinct signaling cascades, the Ras/ERK pathway that mediates differentiation and the AKT-PI3 kinase pathway that enhances survival. Differentiation signaling in PC12 cells induced by NGF through the receptor tyrosine kinase (RTK), TrkA, is well known (Chao et al. 1995). Upon binding to NGF, the receptor TrkA is activated and autophosphorylates itself on Tyr-490, a step which is required for Shc association and subsequent activation of the Ras-ERK signaling cascades. This then leads to activation of Elk-1 dependent gene transcription and neurite outgrowth (Yoon et al. 1997). Significant progress has been made in the identification of the intracellular signaling intermediates that are involved in transducing neurotrophin NGF-mediated neuronal differentiation. However, certain aspects still need to be definitely clarified. For instance, the Ras/ERK cascade has been demonstrated to be both necessary and sufficient for differentiation of PC12 cells stimulated by NGF (Klesse et al. 1999).

However, it was also reported that NGF activates the ERK pathway through not only transient activation of Ras family proteins (Qui and Green 1992; Marshall 1995), but also sustained activation of Rap1, which in turn induces sustained activation of B-Raf, and subsequently the ERKs (York et al. 1998; 2000). Recently, Sun et al. (2006) suggested that NGF induces neuronal differentiation of PC12 cells through sustained activation of M-Ras, one of the isoforms of Ras protein, rather than through sustained activation of Rap1 (Sun et al. 2006). Nevertheless, NGF signaling pathways remains the most established differentiation cascades investigated in PC12 cells (Fig.1.1).

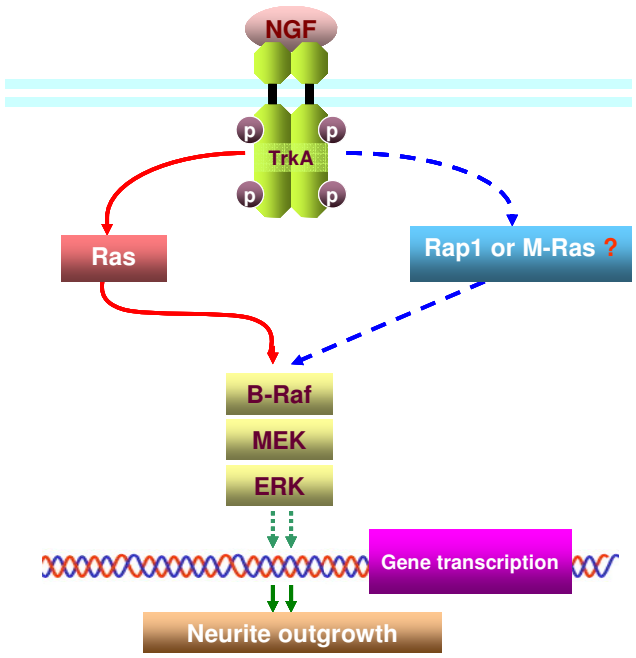


Fig 1.1. Schematic diagram of singaling pathways of NGF-dependent PC12 cell differentiation. Ras and Rap1 (or M-Ras) -dependent signaling are thought to account for immediate and sustained neurite outgrowth effect induced by NGF respectively.

1.1.2. Pituitary Adenylate Cyclase Activating Polypeptide (PACAP) pathway

Following the identification of the neuronal differentiation function of NGF, several reports including Deutsch and Sun (1992), Hernandez et al. (1995) and Vandry et al. (2002) have all shown that treatment of PC12 cells with **p**ituitary **a**denylate **c**yclase-**a**ctivating **p**olypeptide (PACAP) also induces neurite outgrowth and inhibits cell proliferation. The following paragraphs provide a summary of the published works.

1.1.2.1. Pituitary adenylate cyclase-activating polypeptide (PACAP)

PACAP was originally isolated from an extract of ovine hypothalamus on the basis of its ability to stimulate cAMP formation in rat pituitary cells (Miyata et al. 1989). It belongs to a family of peptide hormones that includes secretin, glucagon, growth hormone-releasing factor, and vasoactive intestinal peptide (VIP), and acts via three G-protein-coupled receptors (Harmar et al. 1998). PACAP is the most ancient and conserved member of the VIP/glucagon superfamily. In the last few decades, PACAP has been identified in many vertebrate species, including human (Hosoya et al. 1992; Ohkubo et al. 1992), sheep (Miyata et al. 1989), rat (Ogi et al. 1990), mouse (Okazaki et al. 1995), chicken (McRory et al. 1997), lizard (partial sequence in Pohl and Wank 1998), frog (Chartrel et al. 1991), salmon (Parker et al. 1993,), catfish (McRory et al. 1995), stargazer (Matsuda et al. 1997), and stingray (Matsuda et al. 1998).

The sequence of PACAP is evidently well conserved during evolution from protochordate to mammals, indicating that it is involved in the regulation of primary biological events. There are two isoforms of PACAP: PACAP-27 and PACAP-38. These represent alternatively processed forms of a precursor protein that share a common N-terminal 27 amino acids. PACAP-27 is the more ancient form whereas the vertebrate PACAPs are present as both a 38-amino acid form and a truncated 27-amino acid form. Both forms exhibit substantial amino acid sequence homology with VIP (Miyata et al. 1990) (Fig 1.2). Moreover, the three-dimensional structure of PACAP also exhibits prominent similarities with those of other members of the VIP/glucagon family (Braun et al. 1983; Gronenborn et al. 1987; Wray et al. 1993).

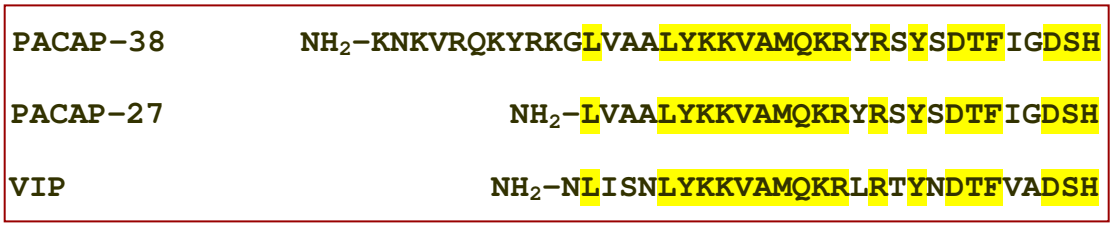


Fig 1.2. Amino acid sequences of the different isoform of PACAP and VIP in human. The amino acid sequences in VIP which are identical with PACAPs are highlighted.

PACAP is widely distributed in the central nervous system (CNS) and peripheral tissues and has been implicated in a broad range of biological processes including reproduction, development, growth, cardiovascular, respiratory, digestive functions, immune responses, and modulation of circadian rhythms (Vaudry et al. 2000). The major form of PACAP in all tissues is PACAP-38, with the highest concentration

found in the hypothalamus, cerebral cortex, hippocampus, posterior pituitary, testes, and adrenal gland (Arimura 1998). In several *in vitro* and *in vivo* models of cerebral ischemia, PACAP-38 has potent neurotrophic and neuroprotective effects (Reglodi et al. 2000; Uchida et al. 1996; Vaudry et al. 2000). PACAP acts as a hypothalamic hormone, neurotransmitter, neuromodulator, and neurotrophic factor that may play an important role during brain development and in learning and memory processes. It has been demonstrated that PACAP-38, when administered intra-cerebroventricularly at very low dosages, improves memory in rat (Sacchetti et al. 2001). In the developing CNS, PACAP expression decreases the number of mitotic cells and enhances neuroblast differentiation (Lu and Dicicco-Bloom 1997). In the adult brain, PACAP regulates neurotransmitter release, inhibits apoptosis (Uchida et al. 1996; Anderson and Curlewis 1998) and is neuroprotective. It reacts to injury, inflammation and may have neural oncogenetic functions through involvement in proliferation/ differentiation processes.

The neurotrophic and neurite-inducing action of PACAP was first observed in PC12 cells in 1992 (Deutsch and Sun 1992). Initiation of neurite outgrowth occurred within 4-8 h after the addition of PACAP-38 to cultured PC12 cells (Lazarovici et al. 1998). Since then, this cell line has been extensively used as a model to investigate the signaling pathways involved in PACAP-induced cell differentiation and survival. The neurite-inducing activity of PACAP-38 is markedly more robust than that observed for PACAP-27 in PC12 cells (Deutsch and Sun 1992). There is clear evidence that

PACAP exerts pleiotropic effects on multiple types of cells. However, many questions remain unanswered with regards to the molecular mechanisms involved in PACAP's effect on proliferation, migration, differentiation, and apoptosis.

Interest in the molecular mechanisms of PACAP-induced neuronal differentiation is especially intense because it represents the prototype for G-protein-coupled receptor (GPCR)-mediated neurotrophins (Waschek et al. 1998; Beaudet et al. 2000; Vaudry et al. 2000; Nicot et al. 2002). NGF, on the other hand, was extensively used as a model for neurodevelopmental processes through the function of tyrosine kinase receptors, (Sofroniew et al. 2001; Torocsik et al. 2002; Glebova and Ginty 2004. These two neurotrophic agents therefore induce neuronal differentiation through distinct receptors and different transduction pathways. They can however synergize to promote the neuronal differentiation and prolong phosphorylation of ERK1 and 2, which are required for the induction of neurite outgrowth (Kao et al. 2001; Sakai et al. 2004). The signal transduction pathways responsible for mediating PACAP-induced neuronal differentiation are not as clearly understood as the NGF-mediated pathway, and their elucidation could reveal fundamentally novel mechanisms responsible for regulating processes associated with morphological development of neurons.

1.1.2.2. PACAP receptors and PACAP receptor type I (PAC1R)

Two classes of PACAP binding sites have been characterized on the basis of their relative affinities for PACAP and VIP. Type I binding sites, which were originally characterized in the anterior pituitary and hypothalamus, exhibit high affinities for both PACAP-38 and PACAP-27 ($K_d \approx 0.5$ nM) and much lower affinities for VIP ($K_d > 500$ nM) (Cauvin et al. 1990; Gottschall et al. 1990, 1991; Lam et al. 1990; Suda et al.1992). Type II binding sites, which are abundant in various peripheral organs including the lung, duodenum, and thymus, however possess a similar affinity for PACAP and VIP ($K_d \approx 1$ nM) (Gottschall et al. 1990; Lam et al. 1990). According to their relative affinity for PACAP and VIP, three PACAP receptors have been cloned, and were termed PAC1R, VPAC1R, and VPAC2R receptors respectively by the International Union of Pharmacology (Harmar et al. 1998). Among these, PAC1R is a PACAP-specific receptor, whereas the VPAC receptors exhibit a similar affinity for both PACAP and VIP (Harmar et al. 1998).

PAC1R was first cloned from a pancreatic acinar carcinoma cell line (Pisegna and Wank 1993) and was subsequently cloned from humans (Ogi et al. 1993; Pisegna and Wank 1996), bovine (Miyamoto et al.1994), rat (Hashimoto et al. 1993; Hosoya et al. 1993; Morrow et al. 1993; Spengler et al. 1993; Svoboda et al. 1993), and mouse (Hashimoto et al. 1996b) sources. The PAC1R cDNA encodes a 495-amino acid polypeptide with seven putative transmembrane domains. It belongs to the G-protein-

coupled receptor (GPCR) superfamily, and exhibits a high degree of sequence identity with the glucagons, secretin and calcitonin receptor cDNAs (Pisegna and Wank 1993). Indeed, PAC1R is the only endogenous GPCR that could stimulate neurite outgrowth in PC12 cells (Tristan et al. 2003). Multiple receptor isoforms are generated by alternate splicing of the PAC1R mRNA. The splice variants are determined by the absence (short variant) or presence of either one or two cassettes consisting of 28 (hip or hop1 variant) or 27 (hop2 variant) amino acids (Journot et al. 1994). The presence of the hip cassette stimulates adenylyl cyclase (AC) only but not phospholipase C (PLC), while the presence of the hop cassette enables activation of both AC and PLC, suggesting that the various cassettes are involved in different second messenger coupling. In the brain and pituitary, the short variant is the most abundant form, whereas the hop variant predominates in the testes and adrenal gland (Spengler et al. 1993). Similarly, a variant of the receptor lacking the fourth and fifth exons encoding sequence in the extracellular domain has been described in hypothalamus and pituitary. This short splice variant of PAC1R is characterized by a 21-amino acid deletion in the N-terminal extracellular region (Pantaloni et al. 1996; Dautzenberg et al. 1999). The existence of this 21-amino acid sequence influences the receptor's ligand binding selectivity for the PACAP-38 and PACAP-27 isoforms and determines the relative potencies of the two peptides in stimulating PLC. Another PACAP receptor variant termed PAC1R transmembrane domain (TM) IV has been cloned in the rat cerebellum and differs from the short variant of the PAC1R by discrete sequence substitutions located in TMs II and IV (Chatterjee et al. 1996).

Surprisingly, activation of PAC1R TM IV has no effect on AC or PLC activity, but causes calcium influx through L-type voltage-sensitive calcium channels (Chatterjee et al. 1996). Other variants of the type I PACAP receptor have also been described (Svoboda et al. 1993; Chatterjee et al. 1996), as well as alternative splicing of the 5'-untranslated region (5'UTR) (Chatterjee et al. 1997). The complex pattern of alternative splicing (as shown in Fig 1.3) modulates its ligand binding and signaling properties, resulting in the fine modulation of PACAP physiological effects (Nicot and DiCicco-Bloom 2001; Lu et al. 1998).

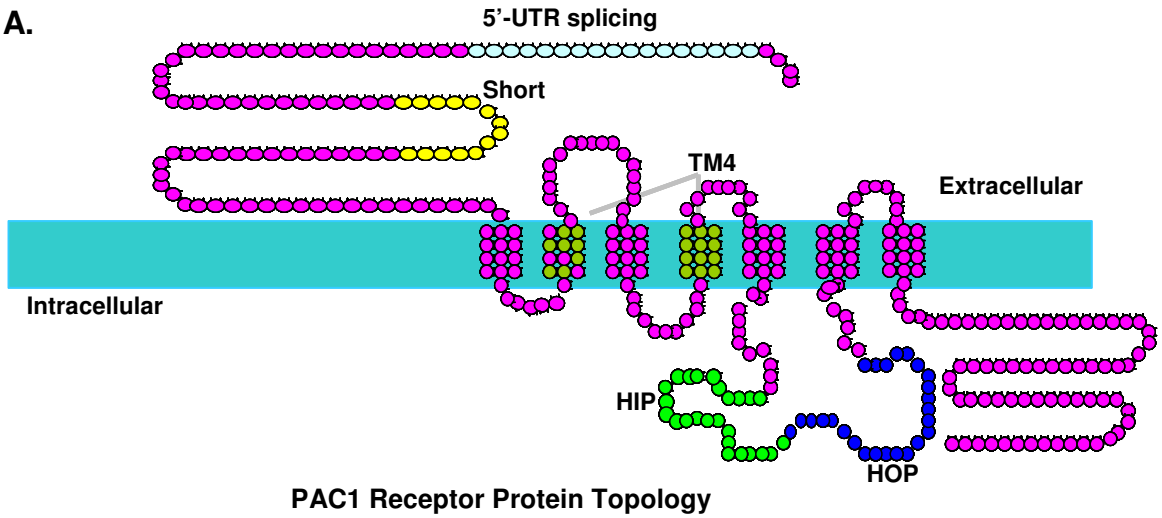


Fig 1.3. A. Putative topology of PAC_1 receptor variant amino acid residues. Alternative splicing in the PAC1R region encoding the amino-terminal extracellular domain and third cytoplasmic loop generates multiple isoforms of the PACAP-selective PAC_1 receptor. The presence or absence of two 83 or 84-base pair exons in the region encoding the third cytoplasmic loop produces receptor variants with the HIP (green) and/or HOP (dark blue) cassettes. Alternative splicing of exons 4 (21 nucleotides) and 5 (42 nucleotides) encodes a 21-residue fragment (yellow) or 5' untranslated region (5'UTR) (light blue) in the extracellular domain of the receptor, producing either short or 5'UTR isoforms. Another PAC1R variant TM4 differs from short variant of PAC1R by discrete sequence substitution in TM2 and 4.

B.

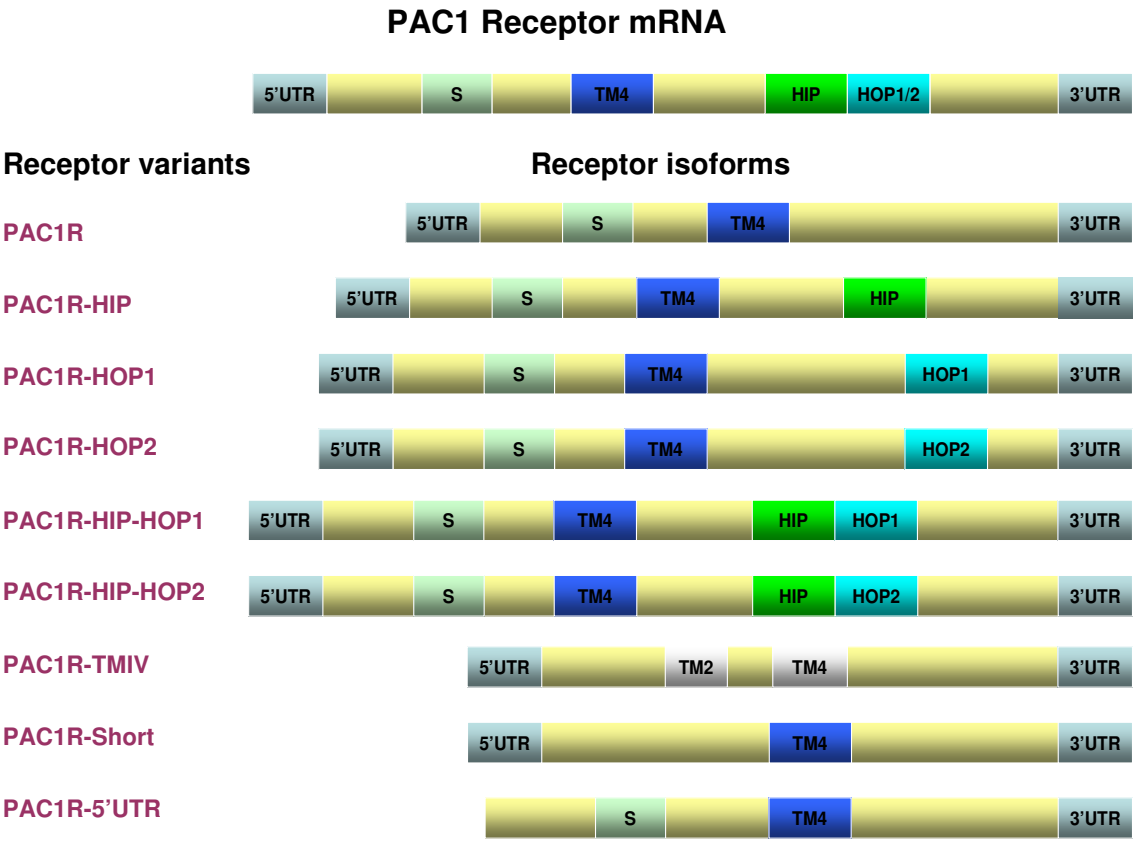


Fig 1.3. B. Schematic diagram of PAC1 isoforms. PAC1 isoforms that differ in either the extracellular N-terminal domains or transmembrane domains (TMs) II and IV or the IC3 third cytoplasmic loop (IC3) are created by alternative splicings.

PAC1R is actively expressed in different parts of neuroepithelia from early developmental stages and in various brain regions during prenatal and postnatal development. In the adult, it exists predominantly in the CNS (most abundantly in the olfactory bulb, thalamus, hypothalamus, the dentate gyrus of the hippocampus and granule cells of the cerebellum) (Spengler et al. 1993). The presence of PAC1R has also been reported in various components of the immune system (Delgado et al. 1996a; Pozo et al. 1997), the cardiovascular system (Gagnon et al. 1994; Adamou et al. 1995; Wei and Mojsov 1996a, b; Wong et al. 1998) and a number of tumor cells such as rat pancreatic acinar AR4-2J (Buscail et al. 1990), medullary carcinoma 6/23 cell line (Vertongen et al. 1994) as well as the human neuroblastoma cell line NB-OK (Cauvin et al.1990; Vertongen et al. 1997a). The latter suggest a possible involvement of PACAP in oncogenesis. Functional PACAP receptors including PAC1R have also been identified in PC12 cells (Watanabe et al. 1990).

The wide distribution of PACAP and PACAP receptors has led to several studies aimed at determining the pharmacological effects and biological functions of the peptide. Analysis of the dynamic interactions of the PACAP with its receptor PAC1R would be rudimental for understanding of the molecular mechanism of the many biological events PACAP is involved in. Furthermore, as PACAP peptides and the PACAP receptors are found to be localized in areas affected by neurodegenerative diseases such as Alzheimer's disease, it may make the investigation of the peptide and its receptors more meaningful in terms of the comprehensive understanding of,

and hence more effective prevention, intervention and therapy of these neurodegenerative diseases.

1.1.2.3. Neurotrophic signaling induced by PACAP

PAC1R is known to be positively coupled to the adenynyl cyclase (AC), PLC and calcium signaling pathways (Hernandez et al. 1995). It can also activate other signaling molecules such as phospholipase D (McCulloch et al. 2001) and Ras (Osipenko et al. 2000). Activation of three PACAP receptors by PACAP typically leads to a G_s-mediated cAMP elevation (Arimura 1998).

It has been recently shown that PACAP-induced differentiation of rat neural stem cells (NSCs) into astrocytes involves the cAMP/protein kinase A (PKA) pathway (Vallejo and Vallejo 2002), while it promotes NSC proliferation via the PAC1R/PKC pathway (Mercer et al. 2004). Thus, it has been suggested that PKC or PKA is involved in PACAP-promoting signal propagation leading to either proliferation or differentiation of neural progenitor cells. However, studies on the PACAP signaling pathways have yielded conflicting findings: It was reported that PACAP induced neurite outgrowth in PC12 cells by increasing cAMP levels (Gunning et. al. 1981), while others suggested that PACAP stimulated both the cAMP and PLC pathways in immature cerebellar granule cells (Basille et al. 1995). PACAP has also been reported to be dependent on PKC but not cAMP-PKA and Ras in inducing neurite outgrowth

in PC12 cells (Lazarovici et al. 1998). The downstream effect of PACAP on the neurite outgrowth is mediated through ERK, a mitogen-activated protein kinase (MAPK), phosphorylation of which is similar to, but distinct from that of NGF signaling (Barrie et al. 1997; Lazarovici et al. 1998; Vaudry et al. 2002b). Furthermore, although NGF (which signals through TrkA) and PACAP pathways may activate several common cellular signaling components, their ultimate effects on gene transcription and cellular phenotype differ substantially (Lazarovici et al. 1998; Vaudry et al. 2002). For example, NGF increases CREB-responsive gene expression through both CREB response element (CRE) and serum response element (SRE) (Bonni et al. 1995), while PACAP increases neuropeptide genes expression through cAMP and calcium signaling pathways (Vandry et al. 2002). In addition, the intensity and duration required for the neurite outgrowth differ in a stimulus-dependent fashion. The small GTPases Ras- and Rap1-dependent signaling through ERK are thought to account for immediate and sustained effects on neurite outgrowth in TrkA-mediated pathways (Qui and Green 1992; York et al. 2000). However, the mechanism of ERK activation in PACAP-induced pathways is still a matter of debate, as some reports indicate that ERK activation requires the PKC pathway (Barrie et al. 1997), while others suggest that cAMP elevation is involved in the stimulation of ERK (Hernandez et al. 1995; Vaudry et al. 2002b). It is also possible that PKA, PKC and Ca^{2+} are all necessary for ERK activation by PACAP in neuronal cells (Bouschet et al. 2003). In addition, it was suggested that PAC1R triggers neuronal differentiation of the PC12 cell line via ERK1/2 activation by transiently activating Ras and inducing the

sustained GTP-loading of Rap1, which is similar to NGF pathway (Bouschet et al. 2003). These different results on the activation of ERK and neuronal differentiation could be explained by the fact that several signaling pathways, including PKA, PKC and the small GTP-loading protein Rap1, may synergize to activate ERK (Bouschet et al. 2003). Alternatively, this could be due to the specific availability of signaling intermediates in different cell lines. In addition, whether the different effects observed were due to PC12 cells cultured in different conditions, ie. in normal serum concentrations or in low serum in which most ERK studies have been conducted (Barrie et al. 1997; Bouschet et al. 2003), are matters which need further clarification. The precise molecular events during PACAP-induced neurite outgrowth and the molecular mechanisms of PAC1R signaling in this regard therefore remain to be fully elucidated. A particular important and interesting aspect of PACAP signaling at the plasma membrane is the membrane microdomains-mediated clustering and organization of receptors and adaptor complexes. The following sections discuss this aspect in more detail.

1.2. Lipid rafts, caveolae and caveolins

Lipid rafts and caveolae are microscopic structures which discovery had changed the way we think about signaling events across cell membranes. These membrane microdomains are small platforms enriched in sphingolipids and cholesterol in the lipid bilayer. They are proposed to exist as laterally segregated regions of cell

membranes that form as the result of selective affinities between certain lipids and membrane proteins. The enriched sphingolipids and cholesterol in these domains act to compartmentalize membrane proteins, separating different biochemical functions (Simons and Ikonen 1997). Caveolae, a unique subset of lipid rafts, are invaginations of the plasma membrane characterized by the presence of the caveolin protein (Anderson 1998). It has been suggested that lipid rafts act in the sorting and subsequent transport of sphingolipids, cholesterol and certain membrane proteins. On the other hand, caveolae have been implicated in clathrin-independent endocytosis as well as in cholesterol efflux. Many signaling molecules are enriched in lipid rafts and caveolae, implicating these microdomains as platforms for assembly and launching of multi-molecular signaling cascades.

In addition, the importance of lipid raft/caveolae signaling in the pathogenesis of a variety of conditions such as Alzheimer's disease, Parkinson's disease, prion diseases, systemic lupus erythematosus, HIV, cardiovascular disease as well as cancer has come to light in recent years (Simons and Ehehalt 2002; Quest et al. 2004). These specific membrane domains are therefore interesting targets for pharmacological approaches in the cure and prevention of those diseases.

1.2.1. The concept and definition of lipid rafts

The original concept of lipid rafts was used to provide an explanation for the directed transport of cholesterol from the *trans*-Golgi to the plasma membrane, formally developed in 1997 by Simons and colleagues (Ikonen and Simons 1997). In 1998, this concept was further related to the liquid-liquid immiscibility observed within model membranes between the liquid ordered phase (Lo phase) and the liquid disordered phase (Ld or Lα phase) (Rietveld and Simons 1998). Although the exact cause remains uncertain, this immiscibility is thought to arise as a result of free energy minimisation of the two phases.

One of the original definitions of lipid rafts is that they differed from the rest of the plasma membrane in being specifically enriched in cholesterol and glycosphingolipids. They are resistant to non-ionic detergents, such as Triton X-100 or Brij-98 at low temperatures (e.g. 4 °C). When detergent is added to cells, it has been hypothesised that the fluid part of membrane liquid will dissolve, while the lipid rafts will remain intact and can be extracted. Based on their composition and detergent resistance, lipid rafts have also been referred to as detergent-insoluble glycolipid-enriched complexes (GEMs), detergent-insoluble glycosphingolipid-enriched membranes (DIGs), detergent resistant membranes (DRMs) or cholesterol-enriched membranes (CEMs) (Dietrich and Jacobson 1999). However, the raft concept has long been controversial, largely because it has been difficult to prove

definitively that rafts exist in living cells. Several studies with improved methodology, such as electron microscopy, florescence resonance energy transfer (FRET) and photonic force microscopy, have dispelled most of the doubts that rafts are detergent-induced artifacts, but rather exist as dynamic microdomains in the membrane of living cells (Brown and London 1997; Harder et al. 1998; Pralle et al. 2000; Varma and Mayor 1998; Friedrichson and Kurzchalia 1998; Wilson et al. 2000). Lipid rafts have indeed been established as critical structures for a variety of cellular processes (Andrew et al. 2004).

1.2.2. The definition of caveolae

Caveolae are specialized lipid rafts that are present in many different cell types (Anderson 1998). These were first described in the 1950s by Palade and Yamada based on their characteristic morphology as 50–80 nm diameter flask-shape invaginations observed by electron microscopy of thin sections (Palade 1953; Yamada 1955). Further study shows that caveolae assume a variety of shapes, including flat, vesicular, and tubular, as patches of membrane with these properties are dynamic domains that assume different shapes depending on their activity in the cell. For instance, flat caveolae that contain caveolin-1 have a striated coat, and become flask shaped during internalization (Smart et al. 1994). They can be either open at the cell surface or closed off to form a unique endocytic/exocytic compartment. In the 1990s, a series of studies provided evidence for the existence of

plasma membrane microdomains enriched in glycosphingolipids, cholesterol, GPI-linked proteins and certain intracellular signaling proteins (e.g. Src family kinases) (Simons and Ikonen 1997). The structure, function, and molecular composition of caveolae are dependent on the phase properties of a unique set of membrane lipids. Resident molecules freely move in and out of caveolae during their lifetime. Purification methods using the caveolae marker protein caveolin established a few criteria for the identification these membrane domains, which includes (*i*) resistance to solubilization by Triton X-100 at 4 °C (Sargiacomo et al. 1993) (*ii*) a light buoyant density (Smart et al. 1995) and (*iii*) richness in glycosphingolipids, cholesterol, and lipid-anchored membrane proteins. The function of caveolae may include cholesterol transport (Smart et al. 1996b), protocytosis (Anderson 1998), signal transduction (Anderson 1998; Li et al. 1996a), and processes that lead to tumor suppression (Galbiati el al. 1998).

Due to the similar lipid composition among lipid raft, caveolae and flattened caveolae, they are co-purified as low density, detergent-insoluble membrane particles. This had contributed to some confusion in the literature with regards to their identity. The distinction among them is still vague, thus making it difficult to define which cellular functions are attributable to rafts and which to caveolae. For example, because cholesterol-modifying drugs such as cyclodextrins and filipin remove cholesterol from plasma membrane rafts and caveolae, these reagents cannot distinguish between the signaling events that occurring in these compartments. To avoid such confusions,

the use of the term "caveolae" is generally reserved for morphologically defined cell surface invaginations (containing caveolins), as originally proposed. Since caveolae are considered to be a subfamily of lipid rafts, lipid rafts and caveolae can indeed be described under a unified concept in many instances.

1.2.3. The composition of lipid rafts/caveolae

It is well known that lipid rafts/caveolae have a specific lipid composition and is enriched in lipid-modified proteins, as well as in receptors and signal-transducing molecules. A more detail description of these components is given below.

1.2.3.1. Proteins in lipid rafts/caveolae

There are three categories of raft-associated membrane proteins: those mainly found in the rafts, those present in the liquid-disordered phase, and those in an intermediate state of flux, moving in and out of rafts. Constitutive raft residents include glycophosphatidylinositol(GPI)-anchored proteins, doubly acylated proteins, such as tyrosine kinases of the Src family, Gα subunits of G proteins, endothelial nitric oxide synthase (eNOS), cholesterol-linked and palmitate-anchored proteins like Hedgehog (Jeong and McMahon 2002), and transmembrane palmitoylated proteins such as influenza virus hemaglutinin and β-secretase (BACE) (Simons and Toomre 2000). This association can at least be partially attributed to the acylated, saturated tails of

both the tyrosine kinases and the GPI-anchored proteins, which matches the properties of sphingolipids more than the rest of the membrane (Simons and Ikonen 1997). Indeed, proteins modified with either GPI or fatty acids are found to be enriched in lipid rafts/caveolae fractions (Sargiacomo et al. 1993; Smart et al. 1995). Mutations that abolish either the GPI-anchor addition (Keller et al. 1992; Ritter et al. 1995) or fatty acid acylation (Robbins et al. 1995; Shenoy-Scaria et al. 1994) shift the protein to other fractions, suggesting that these two different covalent modifications are responsible for targeting proteins with a wide range of biochemical activities to the lipid rafts. When the GPI-anchored complement inhibitor protein CD59 is inserted into the promonocyte cell U937, it is initially dispersed in the membrane but becomes clustered after a brief period. The clustered molecules are active in cell signaling, whereas unclustered CD59 are inactive (van den Berg et al. 1995). Raft targeting is therefore correlated with functional activity.

While these proteins tend to continuously present in lipid rafts, there are others that associate with lipid rafts only when the protein is activated. Some membrane proteins are regulated raft residents and have a weak affinity for rafts in the unliganded state. After binding to a ligand, they undergo a conformational change and/or become oligomerized and may therefore increase their affinity for rafts (Harder et al. 1998). Some examples of these include B cell receptors (BCRs), T cell receptors (TCRs), and an enzyme called CD39 (Horejsí et al. 1999; Matko and Szollosi 2002; Papanikolaou et al. 2005; Petrie et al. 2000). When bound to their ligands, certain

receptors move into lipid microdomains in the outer leaflet of the plasma membrane lipid bilayer. These domains are coupled to microdomains in the inner leaflet that contain signaling kinases, which become activated and initiate signaling cascades (Pierce 2002). Other proteins are completely excluded from rafts, such as transferrin-receptor (Shogomori and Futerman. 2001). There are also proteins that are constantly moving, potentially accessing many different membrane compartments during their lifetime, such as the GPI-anchored folate receptors (Wang et al. 1997). In some cases, protein ligands for GPI proteins shift the receptor proteins from lipid rafts/caveolae to other compartments, where they may become tethered by a resident protein (Stahl and Mueller 1995; Conese et al. 1995; Gliemann et al. 1994; Andreasen et al. 1994). A relatively fast lateral mobility combined with a natural attraction for liquid-ordered phase lipids allows GPI proteins to shuttle information among different membrane compartments. Typically, the raft inclusion or exclusion of proteins is determined by whether or not they are found in membrane fragments extracted using Triton X-100, the 'detergent resistence' defenition of a raft. By these means, the partitioning of proteins in and out of rafts can be tightly regulated.

1.2.3.2. Lipids in lipid rafts/caveolae

Glycosphingolipids, sphingomylin and cholesterol, which form the lipid core of lipid rafts/caveolae, govern the phase properties of the lipids in these domains. Cholesterol is enriched 3- to 5-fold in the detergent-resistant fraction compared to total

membranes, and represents one-third to one-half of the total raft membrane lipid (Brawn et al. 1992; Pike et al. 2002; Prinetti et al. 2002). Cholesterol is thought to serve as a spacer between the hydrocarbon chains of the sphingolipids and to function as dynamic glue that keeps the raft assembly together (Simons and Toomre 2000). Cholesterol compartmentalizes between the raft and the non-raft phase, having a higher affinity for raft sphingolipids than to unsaturated phospholipids. Removal of raft cholesterol leads to dissociation of most proteins from rafts and renders them nonfunctional. One example is that perturbing the lipid-ordered phase with cholesterol-sequestering drugs such as filipin disperses GPI proteins in the plane of the membrane (Rothberg et al. 1990; Smart et al. 1996a). Protein-protein and protein-lipid interactions within lipid rafts/caveolae are the major forces that determine how long the molecules would remain at this site (Zhang et al. 1991; Fiedler et al.1994). The phase properties of the core lipids therefore play major roles in generating the complex molecular environment found in lipid rafts/caveolae.

Sphingomyelin is similarly enriched and represents 10–15% of total membrane lipid in rafts (Brawn et al 1992; Pike et al. 2002; Prinetti et al. 2002). An additional 10–20% of raft lipid comprises glycosphingolipids, such as cerebrosides and gangliosides (Brawn et al. 1992; Prinetti et al. 2002). Glycerophospholipids, including the major membrane phospholipids, phosphatidylcholine and phosphatidylethanolamine, comprise less than 30% raft lipids compared to approximate 60% of lipids in total membranes (Brawn et al. 1992; Pike et al. 2002). Inner-leaflet lipids, such as

phosphatidylethanolamine and anionic phospholipids, are particularly depleted (Pike 2003). Thus Triton X-100 resistant lipid rafts are distinct from bulky plasma membrane because they are enriched in cholesterol and sphingolipids, but are relatively depleted in glycerophospholipids.

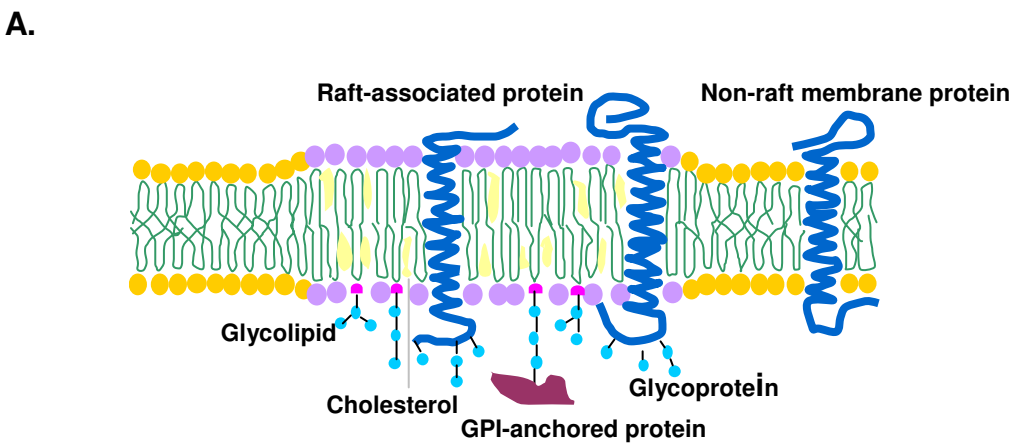


Fig 1. 4. A. Schematic structure of a lipid raft. Lipid raft (purple) is specialized membrane domain containing high concentrations of cholesterol, sphingomyelin, and gangliosides. It is also enriched in phospholipids that contain saturated fatty acyl chains (straight lines in lipid tails). This composition results in lateral phase separation and the generation of a liquid-ordered domain. Bulk plasma membrane (orange) contains less cholesterol, sphingomyelin, gangliosides, and more phospholipids with unsaturated acyl chains. As a result, it is more fluid than lipid raft. A variety of proteins partition into lipid raft: glycosylphosphatidylinositol (GPI) -anchored proteins; transmembrane proteins; glycocylated modification of proteins and lipids. The invaginated caveola, a subclass of lipid raft that contains caveolin is shown as the following diagram (Fig 1.4.B).

B.

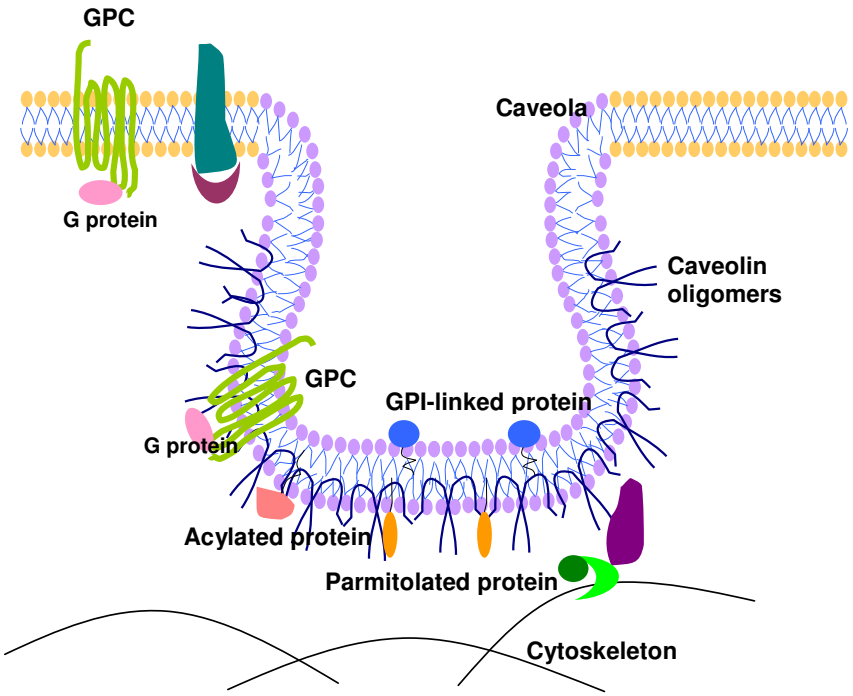


Fig 1. 4. B. Schematic representation of the lipid and protein organization of a caveola. Sphingolipid- and cholesterol-rich domain is shown in purple and nonraft lipid domains are shown in orange. Caveolae contain a coat of oligomeric caveolin molecules inserted into the cytoplasmic leaflet of the membrane. Some proteins, including certain GPCR (shown as heptahelical structures with associated G protein), partition to caveolar domains due to either acylation, binding to caveolin or formation of a sphingolipid 'shell' around the protein or by a combination of these or yet unknown mechanisms. There are also GPI-linked enzymes, receptors (blue) and palmitolated, Src-like kinases (orange) present in the caveolae. Also shown are undefined cytoskeletal interacting proteins (purple, grass green and dark green) and noncaveolar membrane proteins (bottle green) and partners (modena).

1.2.4. Caveolins and their roles in signaling transduction

1.2.4.1 The properties of caveolins

Caveolin is the first marker protein and also the principle protein of caveolae (Rothberg et al. 1992). In the absence of caveolin, no morphologically identifiable caveolae could be observed (Razani and Lisanti 2001). Multiple members of the caveolin gene family have been identified. These include caveolin-1 isoforms -1α (24 kDa 178 amino acids) and -1β (21 kDa, 147 amino acids); caveolin-2 (20 kDa, 149 amino acids), and caveolin-3 (17.2kDa, 151 amino acids) (Way and Parton 1996; Tang et al. 1996; Scherer et al. 1996; Glenney 1992; Kurzchalia et al. 1992). Among them, caveolin-1 was the first discovered and has been most extensively characterized. Caveolin-1 α and -1 β isoforms have in common a hydrophobic stretch of amino acids, the scaffolding domain, and the acylated C-terminal region. An N-terminal 31 amino acids sequence is unique to the α isoform. The two isoforms were shown to have an overlapping but slightly different distribution in mammalian cells (Scherer et al. 1995). No detailed understanding of their functional diversity is available. More recently, three isoforms of caveolin-2 (2α, 2β, 2γ) have been described, however, caveolin-2β, and -2γ have not been characterized (Razani et al. 2002; van Derus et al. 2003). Caveolin-1 and -2 are found in most cells, whereas caveolin-3 is muscle-specific (most abundantly expressed in skeletal and heart muscle cells).

Caveolins are integral membrane proteins and adapt a hairpin-loop conformation in the lipid bilayer. There are three major domains in the structure of caveolins. The scaffolding domain (SCF) (19-21 amino acids in length) is essential for both caveilin oligomerization and the interaction with other proteins (Li et al. 1996a). The membrane-spanning domain (MS) forms a hairpin-like loop within the membrane, thereby exposing both the amino- and carboxyl-termini to the cytoplasmic surface, with no part of the polypeptide chain directly exposed to the extracellular environment (Parton 1996). The caveolin signature motif (CSF) is a conserved sequence (FEDVIAEP) found in all of the caveolins (Razani et al. 2002). Besides the above domains, another feature of caveolin-1 and -3 (but not caveolin-2) is the palmitoylation on three Cys residues at the C-terminal region. The palmitoylation is not necessary for caveolin targeting to caveolae (Dietzen et al. 1995, Bender et al. 2002) but serves to stabilize the caveolin oligomers (Monier et al. 1996). Furthermore, palmitoylation of caveolin has been proposed to have a role in increasing its membrane association mediated by a 33-amino-acid hydrophobic domain (Parat and Fox 2001). The major functional domains in caveolins are illustrated in figure 1.5.

Caveolin isoforms:

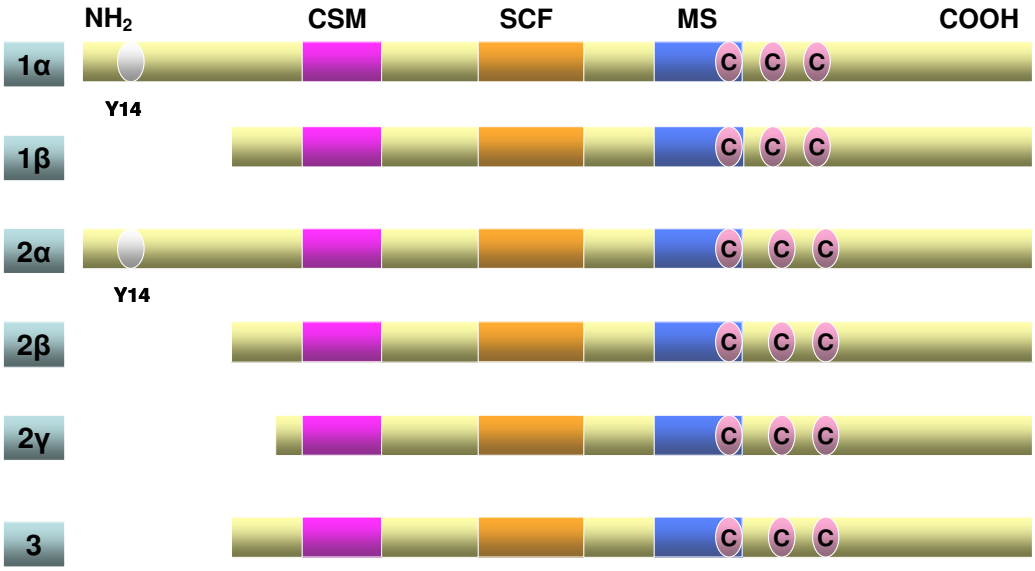


Fig 1.5. Schematic diagram of the domain structure of caveolins. There are three major domains in the caveolins' structure. The scaffolding domain (SCF) is essential for both caveolin oligomerization and the interaction with other proteins. The membrane-spanning domain (MS) forms a hairpin-like loop within the membrane. Caveolin signature motif (CSM) is the conserved sequence for all of the caveolins. Caveolins are parmitoylated on three cysteine residues. Such modifications are likely to stabilize the oligomers and increase its association with the membrane.

1.2.4.2. The cellular functions of caveolins

Caveolin-1 forms oligomers in the membrane, interacting directly with cholesterol, which stabilizes caveolin-1 oligomers (Monier et al. 1996; Murata et al. 1995; Li et al. 1996a). High cholesterol levels in cells cause an increase in caveolin-1 mRNA levels (Fielding et al. 1997). On the other hand, sequestration of membrane cholesterol with drugs such as filipin (Rothberg et al. 1992), or depletion of intracellular cholesterol (Chang et al. 1992), causes the caveolae to disassemble and subsequently disappear. Since there is a threshold level of plasma membrane cholesterol below which caveolae cannot be formed (Hailstones et al. 1998), targeting caveolin-1 and caveolae can be achieved by several methods. These include modulating cholesterol-production and caveolar lipid composition using cholesterol sequestration drugs such as filipin, nystatin, and amphotericin, or cholesterol depletion by β-cyclodextrin, saponin, digitonin, and streptolysin, , or inhibition of cellular cholesterol-biosynthesis by statins (e.g. lovastatin). Direct perturbation of caveolin-1 and caveolae functions by caveolin-1 targeting small interfering RNA (siRNA) is also used in many current studies. Besides, caveolin-1 appears to be part of an intracellular lipid transport system capable of moving sterols between ER and caveolae, and this transport is related to the unique lipidic composition of the caveolar membrane (Murata et al. 1995; Smart et al. 1996b). Furthermore, over-expression of caveolin-1 could lead to *de novo* formation of caveolae (Fra et al. 1995a). These observations suggest that caveolin-1 is an indispensable protein for both the structure and function of caveolae.

Apart from the fact that caveolins have lipid transportation and structural function within caveolae, various signaling molecules found in caveolae interact with caveolins, suggesting that caveolins may also participate in transmembrane signaling. These molecules include receptor tyrosine kinases and their downstream targets (e.g. epidermal growth factor receptor, c-Neu, platelet-derived growth factor receptor, insulin receptor, nerve growth factor receptor, neurotrophin receptor, H-Ras, Raf-1, and ERK), non-receptor protein tyrosine kinases (e.g. Src, Fyn, Yes, Bmx, Btk, and Fak), receptor serine/threonine kinases (e.g. transforming growth factor β-type I receptor), G-protein-coupled receptors and their downstream signaling molecules (e.g. adenylyl cyclase, protein kinase A, β2-adrenoceptors), steroid hormone receptors (androgen and estrogen receptors), and enzymes of nitric oxide (NO) signaling (endothelial and neuronal NO synthase) (Krajewska and Maslowska 2004). Immunoprecipitates of caveolin-1 from cells exposed to insulin (Mastick et al. 1995) or platelet-derived growth factor (PDGF) (Liu et al. 1996) contain different sets of tyrosine-phosphorylated proteins. Anti-caveolin IgG precipitates can also contain eNOS (Feron et al. 1996), Ras (Wu et al. 1997; Li et al. 1997), p75 ^{NTR} (Bilderback et al. 1997), bradykinin receptors (de Weerd et al. 1997), and endothelin receptors (Chun et al. 1994). Immunoprecipitates of alpha integrin (Wary et al. 1996), and dystrophin (Song et al. 1996), contain caveolin-1, whereas caveolin-1 binds Gα (Li et al. 1995), Ras and Src (Li et al. 1996b) *in vitro*. GM1 ganglioside binds caveolin-1 after it is inserted into cells (Fra et al. 1995b). The above receptors and major downstream components in their signaling chains are just a few examples which

suggest that caveolins fulfill a role in the modulation of cellular signaling cascades. The compartmentalization of various signaling molecules in caveolae and their direct and functional interaction with caveolins via the 'scaffold' domain provides a paradigm by which these membrane microdomains are involved in regulating signal transduction pathways.

Caveolins are also implicated in many cellular processes, including transcytosis, vesicular migration, proliferation, apoptosis as well as endocytosis. On the basis of their endocytotic function, they may therefore also be used for drug delivery of new targeted therapeutic agents (Carver et al. 2003; Carver and Schnitzer 2003). In contrast to clathrin-dependent endocytosis, the caveolae-associated internalization pathway avoids lysosomes, and this bypassing of the acidic and disruptive compartment may be a major advantage in drug delivery.

Notably, the association with caveolins seems, in most cases, to be inhibitory and serve to maintain the signaling molecules in an inactive or repressed state (Razani et al. 2002). For example, interaction of caveolins with TrkA and p75 ^{NTR} negatively regulated neurotrophin signaling in neuronal and glial cells (Bilderback et al. 1999). One possible explanation for this could be that the binding to caveolins serves to aggregate the components of a signaling system in a spatially-defined cell circumstances and to prevent inappropriate activation. Once the environment is suitable and the right ligand is available, signaling is then initiated with the molecules

dissociating from caveolins. Some evidence for such a mechanistic notion has been gained in investigation on PDGF and EGF receptors activation (Liu et al. 1996, 1997; Yamamoto et al. 1999; Couet et al. 1997; Mineo et al. 1999). Recent studies show that caveolin-1 can also function as a multi-drug resistance promoter and tumor-promoter, dependent on the tumor type (Cohen et al. 2004;Williams and Lisanti 2005). Thus, the function of caveolin-1 and caveolae continues to be a fertile ground for new discoveries, as well as interesting controversies.

1.3. Signaling transduction from lipid rafts/caveolae

1.3.1. Different signaling transduction pathways from lipid rafts/ caveolae

Lipid rafts/caveolae have gained much attention as important sites for signal transduction. This may be the most important role of rafts at the cell surface in a variety of cell types. Lipid rafts/caveolae containing a given set of proteins can change their size and composition in response to intra- or extra-cellular stimuli. The identification of signaling proteins in lipid rafts/caveolae has led to the hypothesis that these sphingomyelin- and cholesterol-enriched microdomains form a more stable lipid matrix, which can in turn act as an ordered support for receptor-mediated signaling events. The majority of receptors form complex aggregations of effector

molecules, which favors specific protein-protein interactions, thus resulting in the activation of signaling cascades.

Numerous studies support the view above. For example, immunoprecipitation of several GPI proteins co-precipitates protein tyrosine kinases (PTKs) which are reliable markers for lipid raft/caveolae (Stefanov´a et al. 1991). Immunoblotting, enzymatic activity studies, and immunocytochemistry analyses all indicate that lipid raft /caveolae are major locations for PTKs. Their activity has also been localized to lipid rafts/caveolae (Liu et al. 1997). A major substrate for PTKs is, in fact, caveolin-1 (Glenney 1989; Glenney 1986; Glenney and Zokas 1989), in which a peptide sequence (amino acids 82–101) that interacts with c-Src, possibly modulates PTK activity (Li et al. 1996b). In the case of tyrosine kinase signaling, adaptors, scaffolds and enzymes are recruited to the cytoplasmic side of the plasma membrane as a result of ligand activation (Hunter 2000). Both PDGF (Liu et al. 1996) and EGF (Mineo et al. 1996) stimulate the recruitment of multiple signal-transducing molecules to lipid rafts/caveolae, and are functionally connected to effectors in lipid rafts/caveolae. Also they stimulate the migration of the respective receptors out of caveolae. Several studies suggest that caveolin-1 has a role in recruiting G proteins to lipid rafts/caveolae as well as in modulating their activity (Scherer et al. 1996; Song et al. 1996; Li et al. 1996a; Li et al. 1995). In addition, a model of excitation-contraction coupling mediated by lipid rafts/caveolae suggested that calcium storage and entry sites were located at lipid rafts/caveoale (Popescu 1974). Lipid rafts/caveolae play an

immediate role in the biogenesis of the T-tubule system (Parton et al. 1997). Indeed, Ca^{2+} ATPase (Schnitzer et al. 1995; Fujimoto 1993), inositol 1, 4, 5, -trisphosphate (IP3) receptors (Schnitzer et al. 1995; Fujimoto et al. 1992), and calmodulin (Shaul et al. 1996), key molecular components of calcium transport, have all been found to be localized to caveolae. These findings suggest a role for ER-caveolae interactions during calcium signaling and that lipid rafts/caveolae compartmentation at the surface is important for signaling. Numerous biochemical methods (Shaul et al. 1996; Liu et al. 1996; Garcia-Cardena et al. 1996a; Michel and Michel 1997; Sase and Michel 1997) show that the majority of cell surface eNOS is also located in caveolae. This finding suggests that lipid rafts/caveolae are the site of NO production. In another example, GPI 5'-nucleotidase targeted to caveolae may convert extracellular 5'-AMP to adenosine, where it locally activates receptors (Strohmeier et al. 1997).

Last but not least, some of the lipids and lipid-anchored proteins incorporated into rafts in the Golgi apparatus are important sources of signaling intermediates. Substrates for enzymes that release ceramide (Bilderback et al. 1997; Liu and Anderson 1995), IP3 (Hope and Pike 1996; Pike and Casey 1996), and inositolphosphoglycans (IPG) (Clemente et al. 1995; Stralfors 1997) are produced in rafts after a specific stimuli. These responses appear to be specific because neither ceramide nor IP3 is generated in non-rafts fractions, and the IPG released on the extracellular side of the membrane is internalized, presumably by rafts. All these three molecules elicit characteristic cellular responses, which must be a general

mechanism whereby lipids sorted to lipid rafts/caveolae become the source of critical signaling intermediates.

With so many different signaling molecules in one location, lipid rafts/caveolae are the logical places to look for signal integration. For instance, GPI proteins can activate PTKs and generate a Ca^{2+} influx (van den Berg et al. 1995; Morgan et al. 1993). PTKs phosphorylate eNOS, thereby inhibiting the enzyme and promoting its interaction with caveolin-1 (Garcia-Cardena et al. 1996b). However, the released Ca^{2+} will bind calmodulin, which activates eNOS. Any NO produced will stimulate the MAP kinase pathway through Ras, (Lander et al. 1996), in synergy with PTKs (Liu et al. 1997). All the above components are present in endothelial cell lipid rafts/caveolae, allowing cross talk between pathways to occur at one site on the plasma membrane.

Furthermore, the ability of lipid rafts/caveolae to sequester molecules provides an opportunity for locally produced or imported molecules to modulate these signaling events. It has been proposed that one of the functions of the lipid rafts/caveolae is that they form concentration platforms for individual receptors, activated by ligand binding. If receptor activation takes place in a lipid rafts/caveolae, the signaling complex is protected from non-raft enzymes such as membrane phosphatases that otherwise could affect the signaling process. Rafts/caveolae recruit proteins to a new micro-environment, where their phosphorylation states can be modified by local kinases and phosphatases, thus resulting in further downstream signaling. In general,

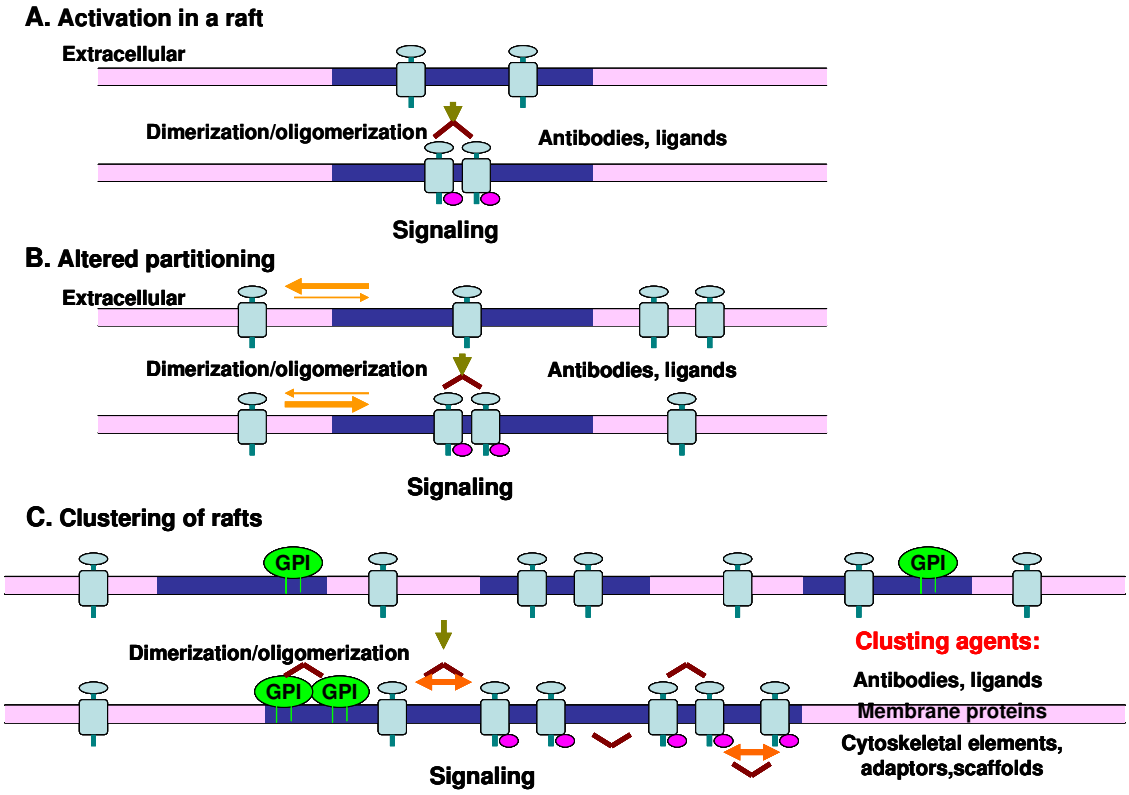
rafts/caveolae represent spatial concentration of specific sets of proteins such that the efficiency and specificity of signal transduction are enhanced by facilitating interactions between proteins and by preventing non-specific cross-talk between pathways (Moffett et al. 2000). However, how these raft receptor complexes form while maximizing spatial requirements by selectively localizing various components from a densely packed cytosolic milieu to satisfy the temporal requirements of cell signaling, is still poorly understood.

1.3.2. Models for signaling initiation through lipid rafts/caveolae

Researchers have tested the presence and importance of lipid rafts/caveolae in cellular signaling based on two models which was proposed to generalize the initial signaling processes (Simons and Toomre 2000) in lipid rafts/caveolae. Signaling initiation occurs either in single raft or clustered rafts. In single raft this can occur by protein activation either within the raft, or by altering the partitioning dynamics of the proteins. The proteins could be phosphorylated in rafts after dimerization or oligomerization. The second model considers the case of having several rafts in the membrane, which differ in protein compositions. Individual raft clusters together to connect raft proteins and interacting proteins into a signaling complex. Clustering could occur either extracellularly, within the membrane, or in the cytosol. It could also occur through GPI-anchored proteins either as a primary or co-stimulatory response. A network of interactions between adaptors, scaffolds and anchoring

proteins would be built up to organize the signal complex in space and time through formation of a raft cluster. The formation of clustered rafts would not only result in the signaling complex being insulated from the surrounding liquid-disordered matrix, but also amplify the signal through concentration of signaling molecules, as well as exclusion of unwanted modulators. The models of signaling initiated by rafts/caveolae are as illustrated in Fig 1.6.

The interactions that drive raft assembly are dynamic and reversible. Raft clusters can be disassembled by negative modulators and/or by removal of raft components from the cell surface. The coalescence of individual raft to form raft clusters has been observed repeatedly when, for example, raft components were cross-linked with antibodies (Harder 1998; Janes et al. 1999). In addition, these two models are not mutually exclusive. For instance, extracellular signals could both increase a protein's raft affinity therefore drawing more of the protein into the raft where it can be activated, as well as recruit other proteins which would crosslink several rafts.



.Adapted with permission from Nature reviews/Molecular cell biology 1,31-9(Oct.2000)

Fig 1.6. Schematic diagram for the model of lipid rafts/caveolae signaling. Signaling occurs in either single raft (A or B) or clustered rafts (C). Following dimerization (or oligomerization) the protein becomes phosphorylated (purple sphere) in rafts. In single raft, this can occur (A) by activation within the raft, or (B) by altering the partitioning dynamics of the protein. In clusters of rafts (C), single raft would be coalesced by clustering so that they would contain a new mixture of proteins such as crosslinkers and enzymes. Raft clustering could occur either extracellularly, within the membrane, in the cytosol or through GPI-anchored proteins (green).

1.4. The importance of lipid rafts/caveolae for GPCR signaling

1.4.1. GPCRs family and their signaling

The GPCRs family with seven transmembrane α-helical domains constitute a large group of membrane receptors known to modulate a wide range of biological responses, including cell growth, differentiation, migration, and inflammatory processes. Their malfunction may lead to a range of pathologies, including psychiatric and neurological disorders. Despite their importance, the mechanisms that regulate their function and signaling remain incompletely understood. Recently, it has become evident that some of the GPCRs, such as angiotensin1 (AT1) receptor, are not homogeneously distributed in the plasma membrane, but instead localizes to lipid rafts/caveolae (Wyse et al. 2003). Human GPCRs have recently been classified into five different groups named GRAFS (an acronym for the groups: **g**lutamate, **r**hodopsin, **a**dhesion, **f**rizzled/taste2, and **s**ecretin (Fredriksson et al. 2003). PAC1R, as discussed earlier, belongs to the secretin subfamily. GPCRs signal through receptors couple to heterotrimeric G proteins. Agonist binding at the receptor leads to exchange of G-protein-bound GDP for GTP. The activated heterotrimer dissociates into its component α subunit and the βγ dimer, both of which have independent capacities to signal downstream through the activation or inhibition of a myriad of effectors. Hydrolysis of GTP to GDP leads to signal termination and re-association of

the heterotrimer. Signal termination is modulated by regulators of G-protein-signaling (RGS) proteins which enhance the weak intrinsic GTPase activity of the Gα subunit.

Under basal conditions, some GPCRs, such as gonadotrophin-releasing hormone (GnRH) receptors are almost exclusively located in rafts (more than 90%) (Navratil et al. 2003). Some others, such as oxytocin receptor (OTR), are present in much smaller proportions (only 10%) (Gimpl and Fahrenholz 2000; Guzzi et al.2002). Each GPCR seems to possess a specific pattern of trafficking to, and association with rafts/caveolae. Some receptors, such as the somatostatin (Sst2) receptor, move into lipid rafts/caveolae upon agonist binding (Krisch et al. 1998; Mentlein et al. 2001). Some receptor moves into lipid rafts after agonist binding in order to activate specific signaling events, but eventually moves out, as in the case of the AT1 receptor (Ishizaka et al. 1998; Wyse et al. 2003). There are also some other receptors that initially reside in lipid rafts but leave after agonist binding, such as the 2-AR (Schwencke et al. 1999; Rybin et al. 2000). These different paradigms reflect the different roles played by lipid rafts/caveolae in regulating the signaling and trafficking of any particular GPCR, and are dependent on cell-specific factors. The precise mechanism of how these receptors specifically shuttling inside/outside rafts remains to be clarified.

1.4.2. The importance of lipid rafts/caveolae for GPCRs signaling

There is growing evidence suggesting that lipid rafts/caveolae actively participate in the regulation of intracellular signal transduction by GPCRs as well as the trafficking of a number of GPCRs. Lipid rafts/caveolae are important for GPCRs signaling because heterotrimeric G proteins, RGS proteins, as well as GPCRs themselves, have been proposed to be targeted to lipid rafts/caveolae. The targeting of GPCRs signaling molecules and the creation of signaling complex in the lipid rafts/caveolae may be dependent on the lipid composition such as fatty acid of lipid rafts/caveolae surrounding the GPCRs and the signaling molecules, which can modulate the targeting of these proteins. For example, Gα subunits are fatty-acylated with amide-linked myristate, thioester-linked palmitate, or both (Wedegaertner et al. 1995), which is proposed to be responsible for the targeting of Gα subunits to lipid rafts/caveolae. The regulator RGS proteins also undergo palmitoylation by an acyl transferase present in the rafts, which is linked to the increase in GTPase activity (GAP) of RGS (Hiol et al. 2003). In addition, GPCRs can also be palmitoylated at specific cysteine residues, which is necessary to target receptors to specific subdomains in the lipid bilayer (Milligan et al. 1995).

The plasma membrane muscarinic acetylcholine receptor, for example, is redistributed to lipid rafts/caveolae upon addition of agonists but not antagonists (Raposo et al. 1987). G-protein-coupled bradykinin B2 receptor was also reported to

induce an upregulation of cellular responses mediated by localization of epidermal growth factor receptor (EGFR) to the rafts. Depletion of cholesterol by methyl-β-cyclodextrin disrupted the raft localization of EGFR and Src, as well as bradykinin-induced signaling. This suggests that lipid rafts/caveolae are essential participants in the regulation of receptor-mediated signal transduction and the crosstalk between different kinds of receptors, presumably via organizing signaling complexes in membrane microdomains (Hur et al. 2004). However, there is much that remains unknown in terms of the dynamic relationship of GPCRs with lipid rafts/caveolae, as well as the regulation mechanisms of both on the signaling transducing process.

1.5. Functions of lipid rafts/caveolae in different biological processes

1.5.1. Lipid rafts/caveolae and immune cell signaling

Much of the information gained about rafts/caveolae was from studies of the immune cell: T lymphocyte, in which rafts are critical for the formation and function of the immunological synapse during initial activation through the T cell antigen receptor (TCR). Some research groups (Marmor and Julius 2001; Goebel et al. 2002; Vereb et al. 2000; Rakesh et al. 2004) have identified lipid rafts/caveolae as an important

component of the interleukin (IL)-2 signal transduction system in T cells. The role of rafts in T cell signaling is not limited to signal transduction in the context of initial activation through the TCR (Leitinger and Hogg 2001; Mañes et al. 2001; Subramaniam et al. 2002). Magee et al. (2002) proposed a mechanism whereby TCR engagement promotes aggregation of lipid rafts/caveolae, which facilitates colocalization of some signaling proteins while excluding others, thereby potentiating protein tyrosine phosphorylation and downstream signaling. Draber and Draberova (2002) have also suggested that the IgE receptor (FcepsilonRI), which is not associated with lipid rafts/caveolae in resting mast cells, induces a weak association with rafts upon aggregation and subsequent activation events. Recent evidence indicates that spatial organization of the immune receptors and components of their signaling cascades are not limited to T cells. Crucial aspects of subcellular localization of signaling molecules in regulation of B cell signaling was also pointed out and discussed (Hassan et al. 2004). Rafts lipid-, protein-controlled interactions and cell biological processes acted synergistically to generate functional domains that mediate B cell activity (Thomas and Karin 2004).

Numerous experiments have provided substantial evidence that raft integrity is crucial for the initiation and maintenance of intracellular signals in lymphocytes. It was found that when rafts were depleted in T lymphocytes, the TCRs lost their ability to relay signals upon antigen attachment (Matko and Szollosi 2002). Similarly, destroying lipid rafts by depleting cholesterol in B cells inhibited signaling through B

cell receptors (BCRs) when they encountered an antigen, and no antibodies were produced (Petrie et al. 2000). It has also been shown that in the absence of rituxan (an anti-CD20 antibody), CD20 exhibits a low affinity to lipid rafts. However, binding of rituxan significantly increases the affinity of CD20 for lipid rafts, and that disturbing the raft integrity by cholesterol extraction results in dissociation of CD20 from lipid rafts followed by complete inhibition of rituxan-induced calcium entry and apoptosis (Janas et al. 2005). All of above suggest an essential role for intact lipid rafts/caveolae for cellular signaling and functioning in the immune-system.

In addition to the immune cells, the function of lipid rafts/caveolae in signaling processes was also investigated in some other cell types. For example, in brain oligodendrocytes, regulation of integrin and growth factor interactions by lipid rafts/caveolae microdomains, through separation or colocalization of integrin and growth factor receptors, generates a signaling environment within the rafts for the activation of survival-promoting PI3K/Akt activity (Baron et al. 2003). More recently, a role for lipid rafts/caveolae in signal transduction mechanisms capable of promoting the neuroendocrine differentiation phenotype in LNCaP PCa (aggressive prostate cancer) cells was also defined (Kim et al. 2004).

1.5.2. Lipid rafts/caveolae and signaling in neuronal cells

Investigations were also focused on the mechanisms of lipid rafts/caveolae in neuronal cell signaling. In 1999, Huang et al. found that many of the intermediates in the signaling cascade activated by Trk receptors which contributed to cell survival were present in lipid rafts/caveolae in PC12 cells, and that neurotrophin NGF binding to $p75^{NTR}$ and TrkA occurred mainly in lipid rafts/caveolae (Huang et al. 1999). Recently, it was reported that in differentiating PC12 cells, the protein tyrosine kinase Pyk2 and multifunctional adaptor protein Cbl, which were implicated in the regulation of the cytoskeleton in several cell types, formed a signaling complex that translocates to lipid rafts/caveolae and were enriched in growth cones of differentiating PC12 cells following growth factor stimulation (Haglund et al. 2004). These results indicated that the recruitment of Pyk2/Cbl complexes to lipid rafts/caveolae participated in growth factor-induced regulation of the actin cytoskeleton in growing neurites.

As a matter of fact, the roles of lipid rafts/caveolae on different neurotrophic factor-induced signaling pathways have gained increasing attention. Ephrins and Eph receptors, the glial cell line-derived neurotrophic factor (GDNF) family receptors (c-Ret and GFR), epidermal growth factor receptors (EGFR), neuregulin receptors (ErbB2-4), fibroblast growth factor receptors (FGFR) were known groups of receptors playing critical roles in neuronal cell survival, proliferation and

differentiation during neuronal development (Wu et al. 1997; Airaksinen and Saarma 2002; Furuchi and Anderson 1998; Trachtenberg and Thompson 1997; Kramer et al. 1996; Gritt et al. 1996). Upon activation, Ephrins, Eph receptors, and neuregulin receptors (ErbB2-4) were reported to localize to the lipid rafts/caveolae microdomains and many signaling events triggered by them appeared to be critically dependent on this localization (Wu et al. 1997; Bruckner et al. 1999; Frenzel et al. 2001). Similarly, GDNF receptors' signaling had been shown to depend on the integrity of lipid rafts/caveolae because cholesterol depletion with methyl-β-cyclodextrin reduced GDNF-dependent activation of MAPK and Akt kinases (Tansey et al. 2000). On the other hand, EGFR concentrated in plasma membrane caveolae under resting conditions, but moved out of caveolae following EGF stimulation (Furuchi and Anderson 1998; Mineo et al.1999). In addition, several rapid signaling events induced by EGF binding, including tyrosine kinase activation, recruitment of adaptor proteins, phospholipase C-mediated hydrolysis of phosphatidylinositol 4, 5-bisphosphate, and activation of Ras/MAPK, appeared to occur within caveolae, suggesting that EGFR signaling was initiated and organized in these microdomains (Mineo et al. 1999). On the other hand, FGFR does not appear to be present in lipid rafts/caveolae. However, many proteins involved in FGFR signaling resided within these lipid domains (Kouhara et al. 1997). For example, stimulation of human neuroblastoma cells with FGF2 was shown to result in tyrosine phosphorylation of several proteins, including the two Src family kinases Fyn and Lyn, as well as Src family substrate anexin II within lipid rafts/caveolae (Davy et al. 2000).

The rapid growth in lipid rafts/caveolae research has brought with it a changing view of these enigmatic membrane domains. Lipid rafts/caveolae constitute a membrane system equal in complexity to any cellular compartment or organelle. Although the role of lipid rafts/caveolae in regulating some transduction pathways is now well established, such as the assembly of the various components of the immune receptor signaling cascade (Dykstra et al. 2001; Sedwick and Altman 2002; Werlen and Palmer 2002), detail understanding the role of rafts/caveolae in the functional regulation of other signaling components is still lacking. Lipid rafts/caveolae are potentially capable of regulating different receptors including GPCRs in different ways depending on the cell's metabolic state, differentiation and stage of growth. As a result, it is difficult to make any generalizations or to elucidate the exact role played by rafts/caveolae in different physiological and pathological conditions.

Although a broad range of receptors has been investigated on these microdomains during these decades; many important aspects of the role of lipid raft/caveolae in neurotrophic factor signaling remain unanswered. In particular, understanding of the roles that lipid rafts/caveolae may play on PACAP-induced neuronal differentiation pathways is largely poor. Furthermore, in specific neurodegenerative diseases such as Alzheimer's disease, these microdomains may be defective, making it important to learn more about their normal biology. From another perspective, lipid rafts/caveolae are important research tools. They clearly contain a variety of signal-transducing

molecules that interact in characteristic patterns after cell stimulation. The ease of lipid rafts/caveolae isolation makes it possible to study how the natural organization of these molecules imparts cell function at the molecular level. The physiological significance of lipid rafts/caveolae in cell signaling is not confined to the cell surface, but is also relevant in several other important processes, such as pathogen entry, receptor internalization, and protein nuclear translocation (Schroeder et al. 2001; Lai 2003).

Previous studies have established that lipid rafts/caveolae are presented in PC12 cells, and they are involved in the propagation of a PACAP-induced signal (Huang et al. 1999; Deutsch and Sun 1992; Hernandez et al. 1995; Lazarovici et al. 1998). In addition, PACAP receptor type I, PAC1R, is expressed and positively coupled to AC6, the type 6 adenylate cyclase isoform found in PC12 cells (Detsch and Sun 1992; Spengler et al. 1993; Oshikawa et al. 2003; Ravni et al. 2006). PC12 cells should therefore serve as an excellent cellular model to study the role of membrane microdomains scaffolded by caveolins, such as those found in neurons (Galbiati et al. 1998; Peiro et al. 2000; Head and Insel 2007), in PACAP-stimulated signaling transduction.

1.6. Objectives of this study

In this study, the main objectives are to unravel the molecular mechanisms of signaling pathways that control the differentiation of neuronal-like PC12 cell upon PACAP stimulation. Since lipid rafts/caveolae have been reported to play very active roles in a variety of biological responses including regulation of the receptors and signaling molecules gathering in NGF-mediated pathways in PC12 cells, they could also be potentially involved in PACAP-stimulated differentiation signaling in this cell line. Therefore, this study aims to gain insight on the putative role of lipid rafts/caveolae and their components, i.e. cholesterol, glycosphingolipids and caveolin, as well as the integrity of lipid rafts/caveolae in PC12 cell neurite outgrowth following PACAP treatment. To achieve these objectives, I used ganglioside GM1 and NB-DNJ treatments to modulate the levels of plasma membrane glycosphingolipids level and cholesterol-methy-β-cyclodextrin complex as exogenous supplementation to increase the level of cholesterol in lipid rafts/caveolae. On the other hand, raft-disrupting agent U18666a was applied to inhibit intracellular cholesterol trafficking, while filipin, nystatin and methy-β-cyclodextrin were used to chelate or deplete cholesterol in the plasma membrane. A gene silencing siRNA approach was used to inhibit the expression level of caveolin-1. Through these methods, the structure of lipid rafts/caveolae microdomains could be perturbed or disrupted and the importance of lipid rafts/caveolae in PACAP signaling can thus be

investigated. This study also aims to identify the precise signaling components and pathways initiated by PACAP, leading to neurite extension in PC12 cells.

Briefly, this study sought to determine:

1). the function of lipid rafts/caveolae in PACAP-stimulated neurite extension process in PC12 cells.

2). the signaling cascades activated upon PACAP agonist binding as well as the molecular mechanisms of the process that lead to neurite generation induced by PACAP in PC12 cells.

3). the underlying molecular mechanisms of how lipid rafts/caveolae modulate PACAP signaling in PC12 cells.

This project may shed light on the fundamental role of plasma membrane lipid rafts/caveolae, especially their core lipids compositions (*ie.* glycosphingolipids and cholesterol) and their integrity, in neuritogenesis upon agonist PACAP stimulation. Also, it may characterize the molecular mechanisms of PACAP-induced signaling transduction pathways mediated by these membrane microdomains, leading to better understanding of the underlying principles of neuritogenesis process. These, in turn, could result in effective prevention and therapeutic intervention of neurodegenerative diseases such as Alzheimer's, Parkinson's disease and promotion of regeneration after CNS injury.

CHAPTER 2. MATERIALS AND METHODS

2.1 Materials

2.1.1. Chemicals

PC12 cell line and horse serum were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA). Cytochalasin D, pituitary adenylate cyclase activating polypeptide-38 (PACAP), direct cAMP enzyme immunoassay kit, RP-adenosine 3',5'-cyclic monophosphorothioate, lithium, methyl-β-cyclodextrin, filipin, nystatin, mouse monoclonal anti-β-actin antibody, crystalline tetramethylrhodamine isothiocyanate (TRITC)-conjugated rabbit anti-goat antibody, poly-d-lysine hydrobromide, dimethyl sulfoxide (DMSO), N, N, N', N'- tetramethyl-ethylenediamine (TEMED), trypsin-EDTA, paraformaldehyde (PFA), phenylmethanesulfonyl fluoride (PMSF), sucrose, sodium dodecyl sulfate (SDS) and valproate were obtained from Sigma (St Louis, MO, USA). Adenosine 4', 5'-cyclic monophosphate, N^6, O^2-dibutyryl (db-cAMP), chelerythrine, FPT inhibitor II, glycine, H89, N - butyldeoxynojirimycin (NB-DNJ), 7S nerve growth factor (NGF) (mouse submaxillary glands), PD98059, 8-(4-chlorophenylthio)-2'-O-methyl-adenosine 3', 5'-cyclic monophosphate (8-OM-cAMP), Triton X-100, U18886A and U73122 were obtained from Calbiochem (Darmstadt, Germany). Opti-MEM® I reduced serum

medium was obtained from Invitrogen Corporation (Grand Island, NY, USA). MESACUP® Protein kinase assay system was purchased from UPSTATE (Lake Placid, NY, USA). Collagen, type I rat tail was obtained from BD Biosciences (Franklin Lakes, NJ, USA). Fetal bovine serum, lipofectamin™ 2000 cationic lipid reagent, RPMI 1640 and Tris glycine SDS running buffer were obtained from Invitrogen life technology (Renfrew, Strathclyde, PA4 9RF, UK). Acrylamide/bis solution, ammonium persulfate, the HRP-conjugated secondary antibodies, the protein assay kit and precision plus protein™ standards were purchased from Bio-Rad Laboratories (Hercules, CA, USA). EZ-Detect™ Rap1 Activation Kit, EZ-Detect™ Ras Activation Kit, Super-Signal® West Femto Maximum Sensitivity Substrate, Super-Signal® West Pico Chemiluminescent Substrate and CL-X Posure™ film were obtained from Pierce (Rockford, IL, USA). The FluorSave™ reagent was purchased from Calbiochem, EMD Biosciences, Inc. (San Diego, CA, USA). The mouse monoclonal transferrin receptor antibody was obtained from ZYMED (S. San Francisco, CA, USA). Rabbit polyclonal caveolin-1 antibody, rabbit polyclonal IgG ERK1/2 antibody, goat polyclonal IgG PACAP receptor antibody and protein G plus agarose were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Phospho-p44/42 ERK1/2 (Thr-202/ Tyr-204) antibody, CREB antibody, phospho-CREB antibody, Elk antibody, phospho-Elk antibody, phospho-GSK3β (Ser-9) antibody, GSK3β antibody, Rap1 antibody, LY294002 and wortmannin were obtained from Cell Signaling Technology (Beverly, MA, USA). Alexa Fluor® 488 and Alexa Fluor® 594 cholera toxin subunit B conjugates, goat anti-rabbit Alexa

Fluor® 488, Hoechst 33342, jasplakinolide and propodium iodide were obtained from Molecular Probes (Eugene, OR, USA). The Complete EDTA-free protease inhibitor cocktail tablet was obtained from Roche Diagnostics (Basel, Switzerland). GM1 Ganglioside was purchased from Avanti Polar Lipids (Alabaster, Al, USA). Re-Blot Plus Strong Solution (10 ×) and Re-Blot Plus Mild Solution (10 ×) were obtained from Chemicon International (Temecula, CA, USA). Absolute methanol and sodium chloride were obtained from Merck (Darmstadt, Germany). Tris (base) and chloroform were obtained from J. T. Baker, Mallinckrodt Baker, Inc. (Phillipsburg, NJ, USA). Bio Trace™ PVDF (polyvinglidene difluoride) transfer membrane was obtained from Pall Corporation (East Hills, NY, USA). Carbon dioxide (CO_2) was obtained from Singapore Oxygen Air Liquid Pte. Ltd. (Singapore). Hydrochloric acid was obtained from Spectrum Chemical Mfg. Corp. (Gaedena, CA, USA).

2.1.2. Instruments and other general consumables

The instruments and general consumables used in this work include biological safety cabinet class II (Gelman Science Inc., Ann Arbor, MI, USA), CO_2 incubator (Heraeus Kulzer Australia Pty Ltd., Chatswood, Australia), eppendorf centrifuge 5810R (B. BRAUN, Melsungen, Germany), TL-100 ultracentrifuge, XL-100 ultracentrifuge, pH meter, SW60 Ti polyallomer microfuge® tube, SW60 Ti rotor and DU®640B spectrometer (Beckman Coulter, Inc., Fullerton, CA, USA), Olympus IX71 fluorescence microscope and Olympus laser scanning confocal microscope (LSM510

and FV 500) (Olympus Technologies Singapore Pte Ltd, Singapore), cyan cytometer (Dakocytomation, Glostrup, Denmark), microplate reader spectraMax 190 (Molecular devices, Sunnyvale, CA, USA), orbital shaker 100 (ARMALAB, Bethesda, MD, USA), Rocker II (Boekel Scientific, Philadelphia, PA, USA), oven and sonicator (Heat Systems, XL 2020, Farmingdale, NY, USA), water bath (Memmert**,** Schwabach, Germany), PowerPac basic power supply 100, mini-gel casting chamber, gel running and transferring system (Bio-Rad Laboratories, Hercules, CA, USA), vortex machine (VWR Scientific, West Chester, PA, USA), ultrasonic water bath (ITS Science and Medical, Singapore), eppendorf tubes (Axygen, Union City, CA, USA), MAX^{TM} 50 ml/ 15 ml polypropylene conical tube, 1 ml / 2 ml / 5 ml / 10 ml / 50 ml nonpyrogenic serological pipette, 22G (0.7 × 38 mm) PrecisionGlide needle, sterile Millex^{TM} syringe driven filter unit (Millipore Corporation, Bedford, MA,USA), 10 ml and 20 ml syringe (Becton Dickinson Company, Franklin Lakes, NJ, USA), Cryo Tube™ vials, 6 well-, 12 well-, 24 well-, 48 well- and 96 well- plate and Easy Flask 75 V/C, 25 V/C (NUNC, Apogent Company, Roskilde, Denmark).

2.2. Cell culture

The transplantable rat pheochromocytoma-derived PC12 cell line was obtained from the American Type Culture Collection (ATCC). It was established from a rat adrenal gland cell. The cells respond reversibly to NGF by induction of a neuronal phenotype

(Greene et al. 1976). PC12 cell adheres poorly to plastic and tends to grow in small clusters. Cell adhesion is improved by using collagen-coated surfaces.

Working cultures were maintained in culture flasks in RPMI 1640 complete medium and incubated in an incubator under a humidified atmosphere containing 5% CO_2 at 37 ºC. The medium was changed every 2-3 days and cells were subcultured when at about 80% confluency.

A new flask was initiated by the following procedures:

1).The complete RPMI 1640 medium was warmed up to 37 ºC before use.

2).The cells were removed from the liquid nitrogen tank and incubated into 37 ºC water bath immediately with gentle shaking. Upon being thawed out, the cells were rapidly transferred into the flask which was prewarmed in the 37 ºC incubator. Another 15 ml RPMI 1640 complete medium was added into the flask. The flask was then placed in an incubator at 37 ºC under a humidified atmosphere containing 5% CO_2 overnight to allow the cells to attach.

3).The medium in the flask was replaced by fresh RPMI 1640 complete medium on the next day after the cells were settled down on the surface of the flask.

4).The cells were passaged after 2-3 days or the cells' confluence reaches 80-90%.

Cells were passaged according to the following procedures:

1).The cells were gently and thoroughly washed with 1 × phosphate-buffered saline (PBS) twice.

2).Two ml of trypsin-EDTA was added to a 75-cm^2 flask for 5 min. The flask was gently shaked or beaten to dislodge the cells.

3).The action of trypsin-EDTA was stopped by adding 10 ml RPMI 1640 complete medium.

4).The cells were resuspended in a new tube and dispersed gently using a 22G PrecisionGlide needle for 10 strokes to dissociate cell clumps.

5).An appropriate amount of the cells were transferred to a new flask or a new plate and appropriate amount of fresh RPMI 1640 complete medium was subsequently added into the flask or plate.

Cells were frozen down according to the following procedures:

Fresh RPMI 1640 complete medium was replaced to cells one day before freezing. Subconfluent cells (70-80%) were trypsinized and centrifuged at 500 g for 5 min at room temperature. The cell pellet was resuspended in 2 ml frozen medium and aliquoted into two NUNC Cryo Tube™ vials. The vials were kept at 4 ℃ for 20-30 min, followed by -20 ℃ for 2 h and then -80 ℃ overnight. The vials were transferred and stored in a liquid nitrogen tank on the following day.

2.3 Cell treatment

To determine the neurite length per cell after different treatments, subconfluent PC12 cells were subcultured and seeded at a density of 0.7-0.8 × 10^3 cells per well in 96-well plates (NUNC) precoated with 50 μg/ml type I collagen. For GM1, cholesterol-methyl-β-cyclodextrin, U18666a compound and NB-DNJ treatments, PC12 cells were pretreated with the above for 24 h before changing to a "serum-starved" RPMI 1640 medium containing 1% horse serum and indicated concentrations of PACAP (or 100 ng/ml NGF) in the presence of the above chemicals. Alternatively, 24 h after seeding, the culture medium was changed to PACAP containing medium in the absence (control) or presence of 8-cpt-cAMP (1 mM), cytochalasinD (1 μg/ml) or jasplakinolide (0.1 μM or 25 nM). Culture was maintained for up to 96 h. For filipin (1 or 10 μg/ml), nystatin (50 μg/ml), methy-β-cyclodextrin (1%), H89 (20 μM), FPTII (0.05 mM), RP-cAMP (0.5 or 1 mM), chelerythrine (10 μM), U73122 (10 μM), PMA (0.1 or 1 μM), PD98059 (100 μM), LY294002 (5 or 10 μM), wortmannin (2.5, 5 or 10 μM), lithium (10 mM), valproate (1 mM) treatments, PC12 cells were incubated with the above chemicals in the RPMI 1640 complete medium for 1 h at 37 °C in the CO_2 incubator. The medium was then removed and changed to fresh serum-starved medium containing PACAP. For gene silencing with siRNA, PC12 cells were transiently transfected with caveolin-1 siRNA, Rap1 siRNA or control siRNA as described in the "Gene silencing" section below, and the medium was changed to fresh RPMI 1640 medium containing PACAP on the following day.

For other biochemical analysis, PC12 cells were seeded into precoated 6-well plate at a cell density of about 6×10^5 cells/well, or 12-well plate at about 3×10^5 cells/well, or 24-well plate at $1\sim2 \times 10^5$ cells/well. 24 h after seeding, the cells were treated with different chemicals as described above. The cells were then washed extensively with PBS before stimulation with PACAP for the desired period of time as indicated.

2.4. Cholesterol-mehtyl-β-cyclodextrin complex preparation

The complex was synthesized as described by Klein et al. (1995) and Emily et al. (2003) with slight modification. Briefly,

1).7.5 mg of free-cholesterol was dissolved in 300 μl of methanol/ chloroform (2: 1 v/v) mixture.

2).0.25 g methyl-β-cyclodextrin was dissolved in 5.5 ml of PBS.

3).This methyl-β-cyclodextrin solution was heated to 80 °C with stirring.

4).The initially precipitating steroid (cholesterol in methanol/chloroform solution) was added in small aliquots to the heated methyl-β-cyclodextrin solution for at least 30 min until complete dissolution.

5).This well melted solution was then aliquot into eppendorf tubes and freeze-dried for at least 24 h to generate the solid cholesterol-methyl-β-cyclodextrin complex.

6).These inclusion complexes were stored at room temperature in a sealed box for further usage.

2.5. Neurite length quantification

1).For neurite length quantification, differently treated PC12 cells were cultured in a humidified 5% CO_2 incubator at 37 °C for as long as 96 h as described.

2).Phase contrast images were acquired using a 20 × objective of an Olympus IX 71 microscope with 1 × adaptor lens of Olympus DP 70 camera at different time points of incubation, i.e.: 24 h, 48 h, 72 h and 96 h respectively.

3).The phase contrast images obtained were changed into 8 bits/channel grayscale pictures using the DP Manager software (Olympus Technologies Singapore Pte Ltd, Singapore) before neurite length quantification.

4).Neurite length was quantified using an interactive semiautomatic neurite tracing technique ("Neuron J") designed and validated by Meijering et al (2004). This technique was implemented in the Java programming language (Sun Microsystems Inc., Santa Clara, CA) in the form of a plugin for ImageJ (National Institutes of Health, Bethesda, MD), the computer-platform independent public domain image analysis program inspired by NIH-Image. The line of each particular neurite can be traced by this "Neuron J" tool precisely. Neurite length was defined as the distance between the cell body and the farthest tip of the neurite. When a neurite reached the margin of the visual field, this was regarded as the end of this neurite. Neurites entering the visual field from outside without origin from a cell body within the visual field were not measured. Since parameters such as percent neurite-bearing cells and mean length of the longest neurite were found proportional to the average neurite

length/cell, only the latter was documented in the figures of this thesis for the sake of explicitness. The total length of neurites was measured and then divided by the number of cells counted to obtain average neurite length/cell.

5). Images of approximately 1000 cells were acquired under each experimental condition. Data were presented as mean ± SD of at least three independent experiments.

2.6. Protein kinase activity assay

To determine the protein kinase A and C activity, the MESACUP® Protein Kinase Assay System (Lake Placid, NY, USA) based on an ELISA method and by making use of a synthetic peptide (RFARKG [pS] LRQKNV) and a monoclonal antibody 2B9 that recognizes the phosphorylated form of the peptide was employed. The peptide was immobilized on the microwell plates and can be phosphorylated by PKA or PKC present in the samples and then recognized by the biotinylated 2B9 antibody, which can be subsequently detected with streptavidin conjugated peroxidase. The color intensity is measured at 490 nm spectrophotometrically using a microplate reader, DU®640B spectrophotometer (Beckman Coulter, Inc., Fullerton, CA, USA).

Protein kinase activity assay was conducted as follows:

1).The reagents which were needed in this assay were prepared as described.

a).ATP (0.1 M): 60 mg ATP was dissolved in 0.8 ml H_2O, pH 7.0 and volume was adjusted to 1 ml with H_2O. The solution was stored at -20 ºC and diluted to 1 mM immediately prior to use.

b).Wash solution: 1 part wash concentrate was diluted with 9 part of distilled H_2O just prior to assay.

c).Substrate solution: one tablet of substrate A was dissolved with 12 ml of substrate B just prior to color development. The solution was kept in the dark and used as soon as possible.

d).cAMP (10 mM): 4 mg cAMP was dissolved in 0.8 ml H_2O and the volume was adjusted to 1 ml with H_2O. The solution was stored at -20 ºC and diluted to 20 µM immediately prior to use.

e).PKA: 0.5 µg of the catalytic subunit of PKA was added to 1 ml of 1 mg/ml bovine serum albumin, 50 mM 2-mercaptoethanol, 50% (w/v) sucrose and 2 mM EGTA. The solution was aliquoted and stored at -70 ºC. The PKA solution was diluted to 0.01-0.05 µg/ml just prior to use.

f).Diluted sample preparation buffer: 50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 10 mM EGTA, 50 mM 2-mercaptoethanol, 1 mM PMSF, 10 mM benzamidine.

g).EGTA (200 mM): 7.6 g EGTA was dissolved in 0.8 ml H_2O and pH was adjusted to 7.0. The volume was topped up to 100 ml with H_2O. The solution was diluted to 20 mM with H_2O just prior to assay.

h).Cell lysate: The subconfluent cells were washed a few times with ice-cold PBS (pH 7.4) and were scraped with rubber scraper. The cells were centrifuged at 500 g

for 5 min. and the pellet fraction was resuspended in 1 ml cold diluted sample preparation buffer. The suspension was sonicated on ice for 30-60 seconds using 15 seconds pulses on and off and then centrifuged at 100,000 g for 1 h at 4 ºC. The cytosolic fraction was saved and used for the protein kinase activity assay.

2).The component mixtures were prepared as describe below:

a).For PKA activity assay, 25 mM Tris-HCl, pH 7.0, 3 mM $MgCl_2$, 0.1 mM ATP, 2 µM cAMP, 0.5 mM EDTA, 1 mM EGTA, 5 mM 2-mercaptoethanol were mixed thoroughly. For PKC activity assay, the 2 µM cAMP in the PKA components was changed to 2 mM $CaCl_2$ and 38 µg/ml PS. The components were mixed thoroughly.

b).108 µl of the above component mixture was added to each well of polystyrene plate and preincubated in a water-bath (23-27 ºC) for 5 min.

3).12 µl of sample was added to each well and mixed thoroughly.

4).100 µl of reaction mixture was added to each peptide-coated well.

5).The plate was incubated in a water-bath (23-27 ºC) for 5-20 min followed by adding 100 µl of stop solution to each well.

6).The solution was removed and the well was washed 5 times with wash solution carefully.

7).100 µl of biotinylated antibody 2B9 was added to each well and the plates incubated in a water-bath (23-27 ºC) for 60 min.

8).The wells were washed again and the wash steps were repeated for 5 times and 100 µl of peroxidase-conjugated streptavidin was added to each well and incubated in water-bath (23-27 ºC) for 60 min.

9).The well was washed and the wash steps were repeated for 5 times and 100 µl of substrate solution was added to each well.

10).The solution was incubated in water-bath (23-27 ºC) for 3-5 min.

11).100 µl of stop solution was added to each well and the optical density of each well was read at 490 nm on the DU® 640B spectrophotometer (Beckman Coulter, Inc., Fullerton, CA, USA). The experiments were performed at least in triplicate.

2.7. Flow cytometry

1). Sample preparation:

a).To quantify the fluorescence intensity of ganglioside GM1 in the plasma membrane by flow cytometry, PC12 cells were incubated with complete RPMI 1640 medium in the absence (control) or presence of 45 µM NB-DNJ or 100 µM GM1 or both in 25-cm^2 flasks for two days.

b).The cells then were washed carefully with ice-cold PBS containing 0.1% bovine serum albumin (BSA; fraction V) and 0.02 M sodium azide (Buffer A) a few times and transferred into eppendorf tubes.

c).The number of the cell was counted by a hemocytometer. The final concentration of the cell was around 1×10^6 cells/ml.

d).The cells were then centrifuged at 500 g for 5 min and washed gently and thoroughly with ice-cold Buffer A for a few times.

e).The supernatant was aspirated.

2). Cell staining:

a).To stain the cells, the pellet fraction of the cells were resuspended in 50 µl PBS/BSA/azide (Buffer A) in the presence of 0.1 mg/ml CTxB-Alexa 488 and were incubated on ice for 30 min in the dark.

b).The cells were then gently washed once with 500 µl buffer A.

c).The cells were resuspended with 1 ml of buffer A containing 2 µg/ml propodium iodide for 15 min on ice.

d).The stained cells in solution were immediately transferred to glass tubes for further analysis.

3). Analysis:

a).The transferred cells were then sorted and counted using a Cyan cytometer (Dakocytomation, Glostrup, Denmark).

b).The data generated were analyzed with the Summit software from Dakocytomation (version 3.3; Fort Collins, CO, USA). At least three independent experiments were carried out.

2.8. Fluorescence and confocal microscopy

2.8.1. Filipin staining

PC12 cells were seeded in 24-well plate which contains a coverslip in each well. The coverslips were double-coated with 1 mg/ml poly-d-lysine and 100 µg/ml collagen type I.

24 h after seeding, PC12 cells were treated with or without (control) U18666a and cholesterol-methyl-β-cyclodextrin for another 24 h.

1).The Cells were washed gently and thoroughly with ice-cold PBS and then fixed with 4% paraformaldehyde (PFA) for 1 h at room temperature.

2).The fixed cells were incubated with 50 µg/ml filipin solution in PBS for 30 min at room temperature.

3).The stained cells were washed with PBS for three times, 5 min each time without shaking.

4).The stained cells on the coverslips were transferred onto glass slide by immersing into anti-fade mounting solution Fluorsave^{TM} Reagent and kept in the dark to be air dried.

5).Digital images were immediately acquired using an inverted microscope (Olympus IX 71) or an Olympus laser scanning confocal microscope system (FV 500).

2.8.2. CTxB staining

To measure the expression level of GM1 in the plasma membrane by fluorescence or confocal microscopy, PC12 cells were treated as previously described.

1).After 48 h's incubation, the medium was removed and the cells were gently washed with ice-cold PBS for several times.

2).PC12 cells were then fixed with 4% PFA for 1 h at 4 °C followed by three times of washing with PBS.

3).After fixation, the cells were stained with 2 μg/ml CTxB-Alexa 594 (diluted in ice-cold RPMI 1640 medium) for 30 min at 4 °C in the dark and followed by several times of washing with PBS.

4).The stained cells on the coverslips were transferred onto glass slide by immersing into Fluorsave^{TM} Reagent and kept in the dark for the sample to be dry before being viewed with an inverted microscope (Olympus IX 71) or an Olympus laser scanning confocal microscope system (LSM 510).

5).Mean fluorescence density was quantified using Image-Pro Plus software (version 4.5.1, Media Cybernetics, Inc., USA).

2.8.3. Immunocytochemistry and colocalization staining

To examine whether of PAC1R receptor and lipid raft/caveolae are colocalized, PC12 cells were stained with both PAC1R receptor antibody and lipid raft/caveolae marker GM1 with fluorescence-conjugated CTxB.

1).The cells were seeded into 24-well plate containing double-coated coverslips at the cell density of 2×10^5 cell/ml and cultured for 24 h before GM1 and NB-DNJ were treated as described previously.

2).The cells were then rinsed with ice-cold PBS a few times and were fixed with 4% PFA for 1 h at room temperature.

3).After a few times rinse with PBS, the cells were preincubated with 10% goat serum in PBS overnight at 4 ºC to block the nonspecific binding.

4).The primary antibody (anti-PAC1R) was diluted with 10% goat serum at the ratio of 1:100 and the cells were incubated with this primary antibody overnight at 4 ºC.

5).The coverslips were washed with PBS for three times (5 min each at room temperature).

6).The rabbit anti-goat TRITC secondary antibody was diluted in the blocking buffer at the ratio of 1:160. The cells were incubated with the secondary antibody for 2 h at room temperature in the dark.

7).The cells were then washed with PBS three times (5 min each at room temperature).

8).Double staining was carried out with 2 µg/ml CTxB-Alexa 488 (diluted in ice-cold RPMI 1640 medium) for 30 min at 4 °C in the dark and followed by several times of washing with PBS.

9).The cells on the coverslips were then mounted before being viewed with the confocal microscope or fluorescence microscope as described previously.

To examine the translocation of PACAP-induced phospho-p44/42 ERK1/2 in cell nucleus after perturbation of lipid rafts/caveolae via caveolin-1 siRNA, PC12 cells were immuno-stained with both phospho-p44/42 ERK1/2 and Hoechst 33342 (direct DNA staining as an indicator of nucleus).

1).Differently treated cells were washed for a few times with PBS and were fixed with 4% PFA for 1 h at room temperature.

2). Fixed cells were permeabilized with 0.1% triton X-100 in 1% goat serum (diluted in PBS).

3).After a few times rinse with PBS, the cells were preincubated with 10% goat serum in PBS overnight at 4 ºC to block the nonspecific binding.

4).The primary antibody (phospho-p44/42 ERK1/2) was diluted with 10% goat serum at the ratio of 1:100 and the cells were incubated with this primary antibody overnight at 4 ºC.

5).The coverslips were washed as describe above.

6).The goat anti-rabbit Alexa Fluor® 488 secondary antibody was diluted in the blocking buffer at the ratio of 1:500. The cells were incubated with the secondary antibody for 2 h at room temperature in the dark.

7).The cells were then washed for three times as described.

8).Double staining was carried out with 1 μg/ml Hoechst 33342 (diluted in PBS) for 15 min at room temperature in the dark and followed by several times of washing with PBS.

9).The cells on the coverslips were mounted and viewed with fluorescence microscope as described previously.

2. 9. Subcellular fractionation

This was performed as described by Wang et al. (2004) with some modifications.

1).Differently treated PC12 cells were harvested and washed with ice-cold PBS and resuspended in hypotonic homogenization buffer (10 mM KCl, 1.5 mM $MgCl_2$, 1 mM Na-EDTA, 1 mM Na-EGTA, 1 mM dithiothreitol, 10 mM Tris-HCl, pH 7.4 with protease inhibitor cocktail).

2).The cells were transferred into 1.5 ml eppendorf tubes and chilled on ice for 20-30 min.

3).The cells were then homogenized carefully with 20 strokes in a Dounce homogenizer.

4).The homogenates were centrifuged at 150 g for 5 min at 4 °C to remove unbroken cells, and followed by 2000 g for 5 min at 4 °C to obtain the pellet of nuclei.

5).The pellet fraction (nuclear fraction) was washed twice with the isotonic homogenization buffer (250 mM sucrose in hypotonic homogenization buffer) and dissolved in the lysis buffer with freshly added protease inhibitor.

6).The fractions were then subjected to SDS-PAGE and Western blot analysis for CREB, phospho-CREB, Elk and phospho-Elk, ERK1/2 and phospho- p44/42 ERR1/2.

2.10. The detergent-soluble and -insoluble sample preparation

This was performed as described by Nyasae et al. (2003) with slight modification.

1).Basically, after different treatments as previously described, PC12 cells were thoroughly washed with ice-cold PBS a few times.

2). The cells were then extracted in 0.3 ml lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1 mM PMSF and protease inhibitor cocktail (Roche)) for 30 min on ice.

3).The samples were homogenized by sonication for 1 min with 15 seconds on-and-off pulses on ice and centrifuged at 100,000 g for 1 h at 4 °C.

4).The resultant pellet fractions were solublized in 0.2 ml solublization buffer (1% SDS, 50 mM Tris-HCl, 5 mM EDTA, pH 8.8) and briefly sonicated for 1 min with alternate 15 seconds of pulsings on and off. The samples were diluted to 1 ml (final volume) with the lysis buffer.

5).The supernatant fractions were also adjusted to 1 ml (final volume) with the lysis buffer, ensuring that both the supernatant (or detergent-soluble) fraction and the resuspended pellet (or detergent-insoluble) fraction would contain the same concentration of buffer components.

6).Identical aliquots of the detergent-soluble and -insoluble fractions from the same sample were taken for immunoprecipitation and immunoblot analysis as described below.

2.11. SDS-PAGE and Western blotting

Samples for SDS-PAGE and Western blotting were prepared according to the following procedure:

1).Differently treated cells were lysed in the lysis buffer with freshly added protease inhibitors on ice.

2).The postnuclear supernatants of cell lysates from each treatment were collected after centrifugation at 13,000 g for 5 min.

3).One volume of 5 × loading buffer was added into four volumes of postnuclear supernatant and the samples were vortexed thoroughly and boiled at 100 °C for 5 min followed by spun at 13,000 g for 2 min.

4).The supernatant of each sample with equal amount of proteins was subjected to electrophoresis using 10 to 15% polyacrylamide gels (Bio-Rad Laboratories).

SDS-PAGE and Western blotting was performed as below:

1).The resolving gel solution was mixed well and allowed to degas before ammonium persulfate (APS) and N, N, N', N'-tetramethyl-ethylenediamine (TEMED) were added.

2). APS and TEMED were added into the resolving gel solution and mixed quickly. The mixture was poured into a mini-gel casting chamber. A depth of 2 cm from the top was left empty.

3).The resolving gel was overlaid with water immediately to separate the resolving gel buffer from air and allowed to stay for 30-60 min.

4).When the resolving gel had solidified, water was removed.

5).APS and TEMED were added into the stacking gel solution (4%) quickly and the mixture was poured on top of the resolving gel.

6). A comb was inserted into the stacking gel immediately.

7).The stacking gel solution was allowed to stay for 30-60 min to solidify.

8).After the stacking gel solidified, the comb was removed.

9).Equal amount of samples and precision protein standard were loaded into each well.

10).The gel was then electrophoresised at constant voltage (40V) while the samples were in the stacking gel.

11).The voltage was adjusted to run at constant voltage (100V) when the dye entered the resolving gel.

12).The electrophoresis was terminated when the dye front reaches the bottom of the resolving gel.

13).The resolving gel was isolated from gel casting chamber and soaked in the precooled transfer buffer for about 10 min.

14).The gel sandwich was stacked in the order of sponge, filter paper, gel, membrane, filter paper and sponge.

15).The proteins were transferred from gel onto PVDF membrane at a constant voltage of 100V for 60 min at 4 °C.

16).After transferring the protein to the PVDF, the membrane was washed with 1 × TBS and then blocked in blocking buffer (5% skimmed milk) overnight at 4 °C or for 1 h at room temperature with shaking.

17).The blots were probed overnight at 4 °C or for 1 h at room temperature with different primary antibodies at indicated dilution with the blocking buffer: anti-PAC1R was diluted to 1:500, anti-Ras was diluted to 1:200, anti-caveolin-1 was diluted to 1:100, anti-ERK1/2 was diluted to 1:3000, anti-phospho-p44/42 ERK1/2, anti-Elk, anti-phospho-Elk, anti-CREB, anti-phospho-CREB, anti-GSK3β, anti-phospho-GSK3β, anti-Rap1, anti-caveolin-1 and anti-transferrin receptor was diluted to 1:1000 and anti-β-actin was diluted to 1:2000.

18).The membrane was then washed with TBST buffer four times to remove the excess primary antibody and then incubated with corresponding secondary antibody conjugated to horseradish peroxidase (Bio-Rad Lab) for 1 h at room temperature.

19).The membrane was then thoroughly washed with TBST buffer four times to remove the excess secondary antibody.

20).The membranes were then submerged in a mixture of equal volume of super signal west pico chemiluminescent substrate and enhancer or in a mixture of equal volume of west femto substrate and enhancer with appropriated dilution with H_2O (Pierce Biotechnology, Inc.).

21).The membrane was removed from the mixture and placed against the film in the cassette.

22).The film was developed using a Kodak X-ray film processor after appropriate exposure.

23).After visualization on film, densitometry of the bands generated was quantified by using Gene Tools software generated on the Chemi-16 Gel DOC machine (Syngene, Frederick, USA).

2.12. Immunoprecipitation

The immunoprecipitation procedure was performed as following:

1).PC12 cells were gently and thoroughly washed with ice-cold PBS for several times.

2).The cells were lysed in the lysis buffer which contains the freshly added protease inhibitor and were chilled on ice for 30 min with occasional vortex.

3).The lysate was centrifuged at 13,000 g for 5 min at 4 ºC.

4).The supernatant fractions were collected and were precleared by adding Protein G Plus-Agarose beads (50% slurry). The mixture was incubated at 4 °C for 10 min on an orbital shaker.

5).Protein G beads were removed by centrifugation at 13,000 g for 10 min at 4 °C.

6).The protein concentration of the cell supernatant fractions was determined.

7).Samples with identical amount of total protein were used for immnoprecipitation and the volume of each sample was adjusted to the same with the lysis buffer. For the detergent-soluble and -insoluble fractions of each sample, whose preparation methods were introduced above, identical aliquots of the membrane fractions were applied for the immunoprecipitation.

8).The same amount of primary antibody (PAC1R) was added to each sample and the mixture was incubated overnight with gentle rolling on a roller at 4 °C.

9).The antibody was then captured by adding protein G Plus-Agarose beads slurry on the next day and incubated for at least 4 h at 4 °C with gentle rolling.

10).The beads were collected by pulse centrifugation at 14, 000 g for 10-30 seconds and washed a few times with ice-cold PBS.

11).The supernatants were discarded.

12).The beads were resuspended with 60 µl-100 µl of 2 × Lameali buffer (loading buffer) and heated at 100 °C for 5 min. The samples were centrifuged and kept at -20 ºC for further usage.

2.13. Gene silencing of caveolin-1 and Rap1 using siRNA

PC12 cells were plated at 50-70% confluence in complete RPMI 1640 cell culture medium in collagen-coated plates and incubated for 24 h. Cells were then transiently transfected in Opti-MEM® I medium (Invitrogen) with Lipofectamine2000 reagent (Invitrogen) and siRNA for caveolin-1 (GeneBank accession number: NM 031556): sense strand CCA GAA GGG ACA CAC AGU UdTdT (5'-3'), and anti-sense strand AAC UGU GUG UCC CUU CUG GdTdT (5'-3'), targeting rat caveolin 221 nucleotides downstream from the start codon, or siRNA for double-stranded RNA oligoribonucleotide sense strand GGC ACC AAC CAU GAA AUC AdTdT (5'-3'), and anti-sense strand UGA UUU CAU GGU UGG UGC CdTdT (5'-3'), which corresponds to the positions 553 to 571 of rat Rap1 (GeneBank accession number: XM-233509), or a scrambled sequence without known homology to mammalian genes as a negative control: sense strand UUC UCC GAA CGU GUC ACG UdTdT (5'-3') and anti-sense strand ACG UGA CAC GUU CGG AGA AdTdT (5'-3'). After 24 h, the Opti-MEM® I reduced serum medium was replaced by RPMI 1640 complete culture medium. Suppression of caveolin-1 or Rap1 expression by RNAi was carried out by Western blotting analysis 48 h after transfection.

Silencing with siRNA was carried out as follows:

1). One day before transfection, PC12 cells were seeded into 6-well or 96-well plate (for neurite quantification assay) according to the following procedures. Cells with

80-90% confluence in culture flask were washed twice with PBS gently. The adherent cells were then detached by trypsinization. The cell suspension was diluted with a 5-fold volume of fresh RPMI 1640 complete medium without antibiotics and transferred to a falcon centrifuge tube. Cells were dispersed by using a 22G needle with 10 gentle strokes. Cells were then seeded into 6-well plate at the cell density of 6 $\times 10^5$ cells/well or into 96-well plate at the cell density of 5×10^4 cells/well so that overnight growth resulted in 70-80% confluence at the time of transfection.

2).24 h after seeding, 1000 µl (6-well plate) or 100 µl (96-well plate) fresh RPMI 1640 complete medium without antibiotics was added into each well before siRNA transfection.

3).The siRNA and Lipofectamine^{TM}2000 complexes were freshly prepared for each transfection.

(i). 4.5 µl (6-well plate) or 0.45 µl (96-well plate) of caveolin-1 siRNA (20 µM), Rap1 siRNA (20 µM) or negative control siRNA (20 µM) was diluted in 250 µl (6-well plate) or 25 µl (96-well plate) Opti-MEM® I reduced serum medium for each treatment condition. The samples were mixed gently, followed by incubation for 5 min at room temperature.

(ii).At the same time, Lipofectamine^{TM}2000 was gently mixed before use. For each treatment, 9 µl (6-well plate) or 0.9 µl (96-well plate) of Lipofectamine^{TM}2000 was diluted in 500 µl (6-well plate) or 50 µl (96-well plate) Opti-MEM® I medium respectively with gentle mix, followed by incubation for 5 min at room temperature.

(iii).After 5 min of incubation, the diluted siRNA was combined with the diluted Lipofectamine^{TM}2000 at a ratio of 1:1. The combination was mixed gently and incubated for 20 min at room temperature to allow the formation of the siRNA and Lipofectamine^{TM}2000 complexes.

4). 500 µl (6-well plate) or 50 µl (96-well plate) of the siRNA or negative control siRNA (for control) and Lipofectamine^{TM}2000 complexes was added to each well which containing 1000 µl (6-well plate) or 100 µl (96-well plate) freshly added RPMI 1640 complete medium without antibiotics, so that the total volume was 1500 µl (6-well plate) or 150 µl (96-well plate) respectively and the final concentrations of siRNA and negative control siRNA were 60 nM. The medium was gently mixed by rocking the plates back and forth. Opti-MEM® I medium was replaced 24 h after transfection with fresh complete medium.

5).48 h after siRNA transfection, analysis on caveolin-1 and Rap1 knockdown efficiency was conducted by Western blotting with caveolin-1 and Rap1 antibodies. β-Actin signals were used as an internal control. The expression level of each protein was determined by densitometry and normalized by comparing with that of β-actin. The results were confirmed by at least three independent experiments.

2.14. Protein determination

Samples for protein determination were prepared according to the following procedure:

1).Culture medium was removed and the ice-cold PBS buffer was used to wash the cells.

2).The cells were lysed and the lysate was collected into eppendorf tubes and chilled on ice for 30 min.

3).Then cell lysate was spun at 13,000 g for 10 min at 4 °C to remove the unbroken cells and nucleus.

4).The postnuclear supernatant was used to determine the protein concentration.

Protein concentration was measured using the RC-DC protein assay kit II from Bio-Rad according to the manufacturer's instructions. A standard curve (0-2 mg BSA/ml) was conducted every time the assay was performed.

1).A total of 25 µl of standard BSA and the samples were added into clean and dry eppendorf tubes.

2).125 µl of RC reagent I was added into each tube which was vortexed and incubated for 1 min at room temperature.

3).125 µl of RC reagent II was added into each tube and the tubes were vortexed and centrifuged at 13,000 g for 10 min at room temperature.

4).The result supernatant was discarded by inverting the tubes on clean and absorbent paper.

5).Liquid was dried completely from the tubes by air.

6).5 µl of DC reagent S was added to each 250 µl of DC reagent A. This solution was referred to reagent A' and 127 µl of reagent A' (prepared freshly) was added to each

tube and the tubes were vortexed and incubated at room temperature for 5 min or until the precipitate was completely dissolved.

7).After the contents were briefly vortexed, 1 ml of DC reagent B was then added to each tube. The tube was vortexed immediately and incubated at room temperature for 15 min.

8).The liquid was transferred into cuvettes and the absorbance of each standard and sample was read at 750 nm on DU®640B spectrophotometer (Beckman Coulter, Inc., Fullerton, CA, USA).

2.15. cAMP enzyme immunoassay

To prepare the sample for the enzyme immunoassay, PC12 cells were subcultured and seeded into 12-well NUNC plate and different treatments were performed as described previously.

The sample preparation was performed according to the following procedure:

1).After treatments, the cells were gently and thoroughly washed with ice-cold PBS a few times to remove the media.

2).The cells were then incubated with 0.1 M HCl for 10 to 20 min until the cells were entirely lysed to stop endogenous phosphodiesterase activity.

3).The cells were scraped carefully into ependorf tubes and centrifuged at 650 g for 5 min at room temperature.

4).The supernatant was directly used in the assay. The protein concentration of the supernatant of each sample was determined and the same amount of protein of each sample was applied for the assay.

5).The volume of each sample was top up to 200 µl with 0.1 M HCl. The sample was ready for the cAMP immunoassay.

A competitive ELISA method was used in this cAMP immunoassay and the procedure follows the manufacture's instruction ((Direct cAMP Enzyme Immunoassay Kit from Sigma). A polyclonal antibody to cAMP was used to bind, in a competitive manner, cAMP in the sample or an alkaline phosphatase molecule conjugated with cAMP. Samples or standards, alkaline phosphatase conjugate and antibody were simultaneously incubated at room temperature in a secondary antibody-coated microwell plate for 2 h with constant shaking on an orbital shaker. The reagents in excess were then washed away and substrate was added and the mixture was incubated at room temperature without shaking for 1 h. The enzyme reaction was then stopped and the yellow color generated was spectrophotometrically measure with a microplate reader at 405 nm. The intensity of the bound yellow color was inversely proportional to the concentration of cAMP in either standards or samples. The measured optical density was used to calculate the concentration of cAMP as described below.

The cAMP enzyme immunoassay was performed according the following procedure:

1).The 2000 pmol/ml cAMP standard solution was warmed to room temperature. Series dilution to the following concentrations: 20, 5, 1.25, 0.312 and 0.078 pmol/ml respectively was prepared as the standards.

2).To increase the sensitivity of the assay, the samples and standards were acetylated by adding in acetylating reagent (1 part of acetic anhydride with 2 parts of triethylamine). Ten µl acetylating reagent was added for each 200 µl of standard or sample. 50 µl of acetylating reagent was added to the Zero Standard/NSB (none specific binding). The acetylating reagent was added directly to the samples or standards and the mixtures were vortexed for 2 seconds.

3).50 µl of the Neutralizing Reagent was added into each well, except the TA (total activity) and Blank wells.

4).150 µl of the 0.1 M HCl was pipetted into the NSB and 100 µl of the 0.1 M HCl was pipetted into the B_0 (0 pmol/ml standard) wells.

5).100 µl of the standards and samples was added into the appropriate wells.

6).50 µl of cAMP conjugate was added into each well except the TA and Blank wells.

7).50 µl of cAMP antibody was added into each well, except the Blank, TA and NSB wells.

8).The reaction plate covered by the plate sealer provided was incubated at room temperature for 2 h on a plate shake at 250 rpm.

9).The contents of the wells were removed and the well was washed three times by adding 200 µl of wash solution to every well. After the final wash, the wells were

emptied thoroughly and the plate was firmly tapped to remove any remaining washing buffer.

10).5 µl of the cAMP conjugate was added to the TA well.

11).200 µl of the p-NPP substrate solution was added to every well and the mixture was incubated at room temperature for 1 h without shaking.

12).50 µl of stop solution was added to every well to stop the reaction and the optical density of the sample was read immediately at 405 nm on a plate reader.

13).The results can be calculated as follows:

Average Net Optical Density = Average bound OD- Average NSB OD

Percentage bound= Net OD/ Net B_0 OD

A logit-log paper was used to plot percent bound (B/B_0) versus concentration of cAMP for the standards. A straight line through the standard points was approximated accordingly. The concentration of cAMP in the unknown samples can be determined by interpolation.

2.16. Sucrose density gradient centrifugation

The cell extract for the continuous sucrose gradient was prepared as following:

The cell extract was washed with ice-cold PBS buffer a few times and scraped into the homogenization buffer (250 mM sucrose, 3 mM imidazole, 1 mM EDTA, with freshly added protease inhibitor pH 7.4) at 4 ºC. The extract was sequentially disrupted by homogenization with a Dounce homogenizer (20 gentle strokes). The

homogenate was then centrifuged at 2700 rpm for 10 min at 4 ºC. The supernatants were applied to the continuous sucrose gradient.

To make the 10–40% continuous sucrose gradient, 10% and 40% sucrose in homogenization buffer was allowed to diffuse into continuous gradients. The volume of the 40% and 10% sucrose solutions to be added was marked according to the standard marker which was provided on the tube rack by Beckman. The 40% and 10% sucrose solutions were then layered into Beckman SW61Ti polyallomer microfuge tubes subsequently and diffusion was allowed to proceed.

The procedure was carried out as following:

1) The lighter sucrose, 10% solution was layered in a centrifuge tube which should be placed straight in a tube rack.

2).A denser sucrose solution (40%) was carefully overlaid to the lighter sucrose solution. The interface would be formed at this point.

3).The tube was closed with a silicon rubber stopper.

4).The tube rack on its side was gently laid down, and allowed to diffuse for 4 h.

5).The tube racket was slowly put to the upright position. The gradients were formed and ready to use.

6).The prepared samples were then layered onto the top of the sucrose solution.

7).Equilibrium centrifugation was carried out on the 10-40% continuous sucrose gradient.

8).Samples were then centrifuged in a SW61 Ti rotor in XL-100 ultracentrifuge (Beckman Coulter, Palo Alto, CA) at 30,000 rpm for 16 h at 4 ºC.

9).After centrifugation, ten fractions were carefully collected from top to bottom of the tubes and aliquots were subjected to SDS-PAGE and Western blotting with Rap1 and TfR antibodies.

2.17. Ras and Rap1 activation assay

Differently treated cells as described previously were collected and used for Ras and Rap1 activation assay.

To evaluate Ras activation, a GST-fusion protein containing RBD of Raf1 was used to specifically pull-down active Ras. GST-Raf1-RBD was incubated with cell lysate in the presence of a SwellGel® Immobilized Glutathione Disc. In Rap1 activation assay, the GST-RalGDS-RBD fusion protein was incubated with cell lysate and a Swell Gel® Immobilized Glutathione Disc. The pulled-down active Ras and Rap1 were detected by Western blot analysis using anti-Ras antibody and anti-Rap1 antibody respectively.

The procedures were carried out following manufacturer's instruction:

1).The medium was removed and the cells were rinsed by ice-cold TBS.

2).0.4-0.6 ml Lysis/Binding/Wash buffer per 6-well plate was added to lyse the cells.

3).The cells were scraped and transferred to a new microcentrifuge tube and incubated on ice for 5 min.

4).The cells were then centrifuged at 16,000 g at 4 ºC for 15 min.

5).The supernatant was transferred to a new tube.

6).The protein concentration was determined before performing the activation assay.

7).The SwellGel® Immobilized Glutathione Disc was placed into a spin column with a collection tube.

8).80 µg of GST-Raf1-RBD was added to the disc containing spin cup for Ras activation assay, while 20 µg of GST-RalGDS-RBD was added to the spin cup for Rap1 activation assay.

9).Equal amount of cell lysate (up to 750 µl) for different treatments was immediately transferred to the spin cup and the sample was vortexed.

10).The cap of the collection tube was sealed with Parafilm® laboratory film to prevent the leakage from the cap and the sample was vortexed.

11).The reaction mixture was incubated at 4 ºC for 1 h with gentle rocking.

12).The film was removed and the spin cup was transferred to a new collection tube.

13).The resin was washed by adding 400 µl of Lysis/Binding/Wash buffer to the resin. The buffer was reverted for three times and the sample was centrifuged at 7200 g for 10-30 seconds. The buffer was decanted.

14).The above washing step was repeated for two more times.

15).The spin cup was transferred to a new collection tube.

16).60 µl sample buffer was prepared for each pull-down reaction by mixing 1 part β-mercaptoethanol to 20 parts 2×SDS sample buffer.

17).The above sample buffer containing β-mercaptoethanol was added to the resin. The cap was wrapped with laboratory film and boiled at 100 ºC for 5 min.

18).The tube was centrifuged at 7200 g for 2 min. Samples were stored at -20 ºC until use.

2.18. Statistical analysis

Statistical analyses were performed using the SPSS statistical package (version 11.0, SAS Institute, Chicago, IL, USA). Data were presented as mean ± SD. For statistical analysis, one-way ANOVA followed by Turkey's post-hoc tests was applied. $p<0.05$ was considered statistically significant. In the figures and figure legends, $p<0.05$ was indicated with an *, **$p<0.01$, ***$p<0.005$.

CHAPTER 3. LIPID RAFTS/CAVEOLAE IN PACAP-INDUCED NEURITOGENESIS IN PC12 CELLS

3.1. Introduction

As alluded to in Chapter I, lipid rafts/caveolae are plasma membrane microdomains enriched with both glycosphingolipids and cholesterol (Brown and London 1998a). Cholesterol regulates both the flexibility and mechanical stability of the membrane bilayer and plays a critical role in assembling membrane microdomains. It is thought to function as a spacer between the sphingolipid hydrocarbon chains and to serve as a glue to maintain the raft assembly (Simons and Toomre 2000). Removal of raft cholesterol leads to disruption of the integrity of lipid rafts/caveolae and dissociation of most proteins from the membrane microdomains (Simons and Ehehalt 2002). The most effective way of disrupting caveolae function therefore involves the use of sterol-binding drugs that sequester cholesterol. In fact, depletion, redistribution, or removal of this lipid results in the flattening and disassembly of caveolae and unclustering of receptors (Schnitzer et al. 1994; Rothberg et al. 1990; 1992). Such sterol-binding drugs include nystatin, filipin and methyl-β-cyclodextrin (CD) (Orlandi and Fishman 1998; Schnitzer et al. 1994; Rothberg et al. 1992; Neufeld et al. 1996). Cholesterol-depleting reagents, such as saponin has also been used to destabilize DRM structures, rendering them sensitive to detergents such as Triton X-100 (Wassler et al. 1987; Montixi et al. 1998). On the other hand, cholesterol intracellular

trafficking inhibitors, such as U18666a (Bierkamper and Cenedella 1978), which impairs transport of cholesterol to the plasma membrane (Liscum and Colliiins 1991; Sexton et al. 1983), are also effective in depleting rafts cholesterol.

In addition to cholesterol, glycosphingolipids, which contain long and largely saturated acyl chains pack tightly in the plasma membrane and are a key component of lipid rafts/caveolae organization (Brown and London 1998b; Ahmed et al. 1997; Schroeder et al.1998). It is believed that these carbohydrate-containing lipids could also affect the integrity and stability of rafts/caveolae (Simons et al. 1999; Mitsuda et al. 2002; Liu et al. 2004). Hence, the integrity of the lipid rafts/caveolae is expected to be compromised by alteration of the cellular glycosphingolipid level. This can be achieved by several approaches, such as by administration of exogenous gangliosides (Farooqui et al. 1997; Simons et al. 1999; Crespo et al. 2002), overexpression of a glycosphingolipid synthase gene (Mitsuda et al. 2002; Nishio et al. 2004) or by inhibitors of glycosphingolipid biosynthesis (Platt et al. 1994a; b; Nagafuku et al. 2003). In this study, the ganglioside level was modulated by exposure of the cells to exogenous GM1 or treatment with NB-DNJ, a potent inhibitor of glucosylceramide biosynthesis (Platt et al. 1994a; b).

3.2. Results and discussion

3.2.1. PACAP-induced neurite outgrowth in PC12 cells is attenuated by perturbation of the integrity of membrane lipid rafts/caveolae

3.2.1.1. Inhibition to the biosynthesis or intracellular transport of the major lipid components of lipid rafts/caveolae, glycosphingolipids and cholesterol, inhibits PACAP-induced neurite outgrowth in PC12 cells

PACAP-stimulated PC12 cells were treated with NB-DNJ or U18666a at different concentrations for 24 h before phase contrast micrographs of the cells were taken for the following 4 days at indicated time points (i.e. 24 h, 48 h, 72 h and 96 h). The average neurite length per cell was quantified as described in Materials and Methods, and the relative change under various conditions was shown in Fig 3.2.1.1.A. Both NB-DNJ and U18666a inhibited PACAP-induced neurite outgrowth in a dose-dependent manner. The maximum inhibitory effect of NB-DNJ on the neurite length reached approximately 80% of that induced by PACAP alone. On the other hand, although the inhibitory effect of U18666a at the indicated concentrations (70 nm, 500 nm,1 μM) was not as strong as that of NB-DNJ at the concentrations applied (45 μM, 90 μM, 180 μM), the maximum repressive effect of U18666a on neurite length also reached 70% of that induced by PACAP alone. These results demonstrate that

reduction of the plasma membrane glycosphingolipid or cholesterol level by NB-DNJ or U18666a attenuated PACAP-induced neurite outgrowth in PC12 cells. It appears that the maximal inhibitory effects of NB-DNJ and U18666a were achieved after the

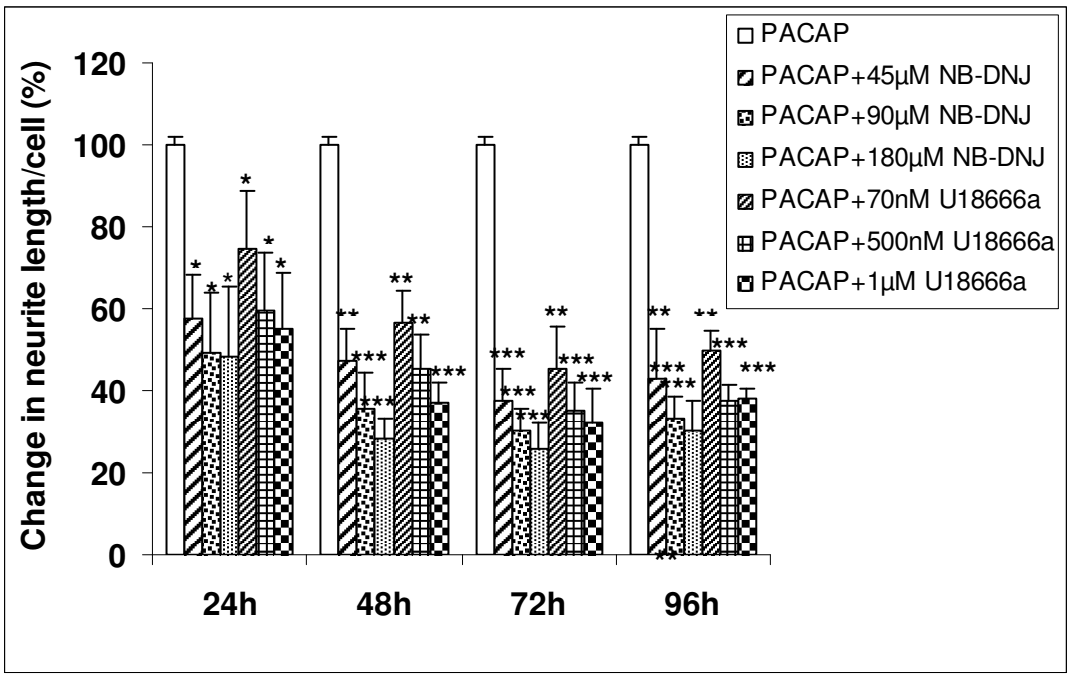


Fig 3.2.1.1.A. The inhibitory effects of NB-DNJ and U18666a on the neurite outgrowth induced by PACAP in PC12 cells. PC12 cells were pretreated with exogenous NB-DNJ at various concentrations (45, 90 or 180 μM) or U18666a at different concentrations (70, 500 nM, or 1 μM) for 24 h. The average neurite length/cell was measured at time points of 24 h, 48 h, 72 h and 96 h after PACAP (100 nM) was added. The relative change was expressed as a percentage of the mean neurite length/cell for the cells treated with PACAP alone (100%) as described in Materials and Methods. The results are shown as means ± SD for at least three independent experiments. * $p< 0.05$, ** $p< 0.01$ and *** $p< 0.005$ versus the samples treated with PACAP alone for the same period of time.

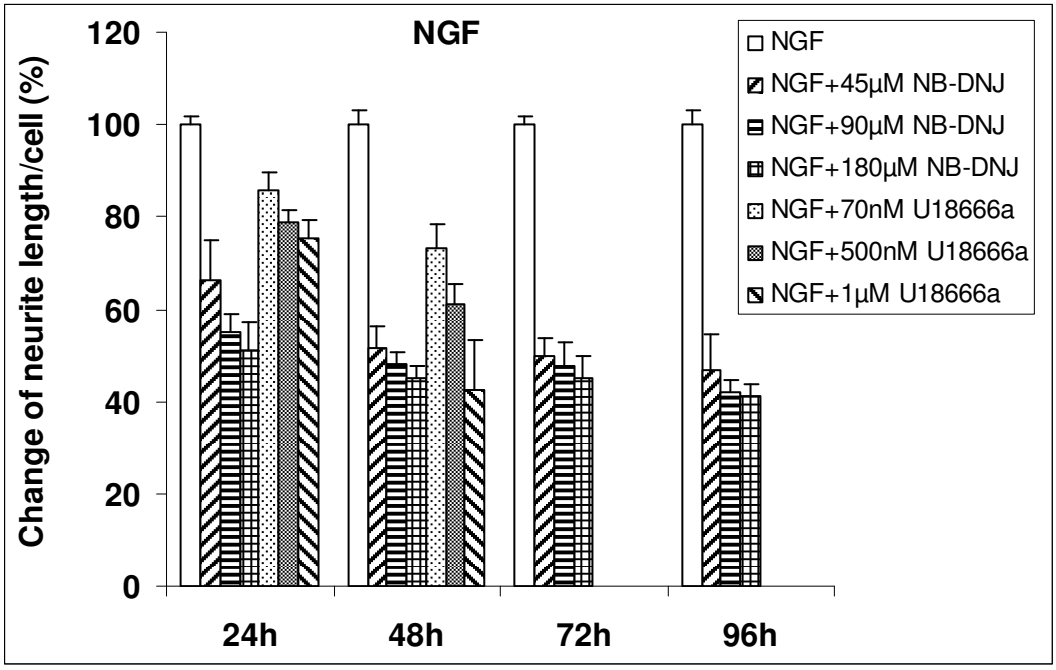


Fig 3.2.1.1.B. The inhibitory effects of NB-DNJ and U18666a on the neurite outgrowth induced by NGF in PC12 cells. PC12 cells were pretreated with exogenous NB-DNJ at various concentrations (45, 90 or 180 μM) or U18666a at different concentrations (70, 500 nM, or 1 μM) for 24 h. The average neurite length/cell was measured at time points of 24 h, 48 h, 72 h and 96 h as indicated after NGF (100 ng/ml) was added. The relative change was expressed as a percentage of the mean neurite length/cell for the cells treated with NGF alone (100%). The results are shown as means ± SD for at least three independent experiments.

cells were treated for 48 h to 72 h. A similar inhibitory effect by NB-DNJ and U18666a on neurite outgrowth was also seen in NGF-induced PC12 cells (Fig 3.2.1.1.B). Therefore, decrease in the two major lipid components of lipid rafts/caveolae, glycosphingolipids and cholesterol, could attenuate neurite outgrowth of PC12 cells induced by both NGF and PACAP, suggesting that lipid rafts/caveolae microdomains might be essential for neurite outgrowth of PC12 cells.

3.2.1.2. Drugs targeting at cholesterol in the plasma membrane retards PACAP-induced neurite outgrowth in PC12 cells

To further address the importance of membrane rafts/caveolae in PACAP-induced cell differentiation, the effects of cholesterol-chelators nystatin, filipin and methyl-β-cyclodextrin (CD) on neurite outgrowth of PC12 cells were investigated. In the control experiments, the mean neurite length per cell reached 28.3 ± 3.3 μm after PC12 cells were cultured for 24 h in the presence of PACAP. Pretreatments with nystatin (50 μg/ml), filipin (10 μg/ml) or CD (1%) for 1 h retarded the PACAP-induced neurite outgrowth by approximately 50% (Fig 3.2.1.2). The inhibitory effects of these drugs were attenuated slightly 48 h after the cells in culture, but the difference in the mean neurite length per cell between the drug-treated and the control cells was still significant (about 30% retardation). This attenuated inhibitory effect of the cholesterol chelators on neurite outgrowth after 48 h's treatment could be due to the compensation mechanisms of the cells that may synthesis or recruit more rafts

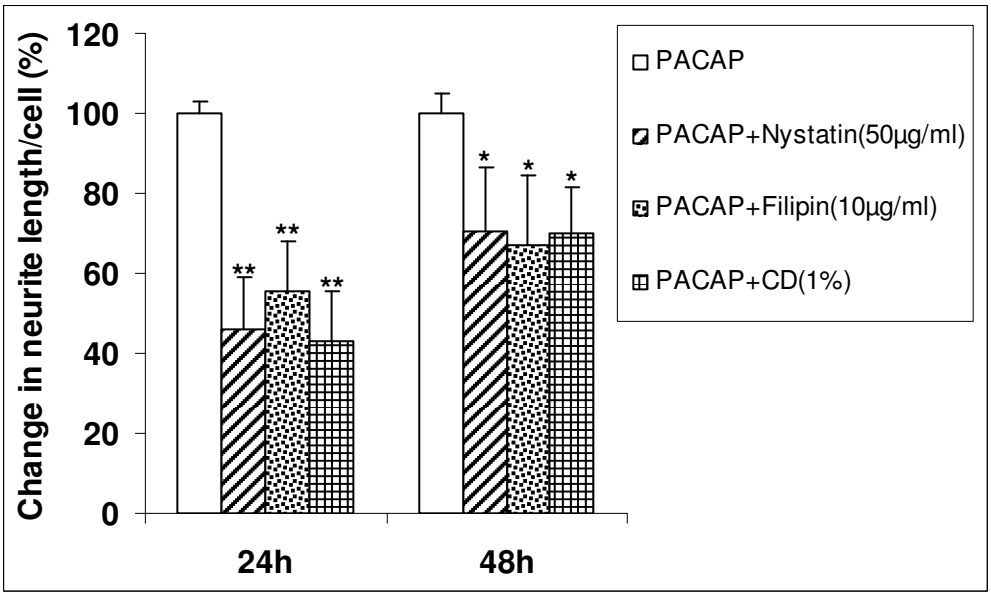


Fig 3.2.1.2. Effect of cholesterol sequestering agents: nystatin, filipin or cyclodextrin (CD) on PACAP-induced neurite outgrowth in PC12 cells. PC12 cells were pretreated with nystatin (50 µg/ml), filipin (10 µg/ml) or methyl-β-cyclodextrin (CD) (1%) for 1 h. The mean neurite length/cell was measured at 24 h and 48 h in the presence of PACAP (100 nM), and the relative change was expressed as a percentage of the mean neurite length/cell for cells treated with PACAP alone (100%), as described in Materials and Methods. The results are shown as mean ± SD for at least three independent experiments. * $p < 0.05$ and ** $p < 0.01$ versus the samples treated with PACAP alone for the same period of time. The results are shown as means ± SD for at least three independent experiments.

lipids to these microdomain after prolonged incubation time (i.e. 48 h). The results obtained here provided compelling evidence that perturbation of the integrity of c cholesterol and glycosphingolipid-sensitive microdomains, such as lipid rafts/caveolae, affected the neurite outgrowth in PC12 cells, supporting the importance of lipid rafts/caveolae in neuritogenesis process in PC12 cells.

3.2.1.3. Caveolin-1 siRNA attenuates PACAP-induced neurite outgrowth in PC12 cells

To ascertain that caveolae function specifically as a signaling platform for this process, the expression of caveolin-1, the major structural protein of caveolae, was silenced by caveolin-1 siRNA. As shown in Fig 3.2.1.3.A, PC12 cells transfected with caveolin-1 siRNA showed a marked reduction (≈70%) in the expression level of caveolin-1 protein compared to untreated cells, or cells transfected with a control siRNA of random sequence. As a result, neurite outgrowth was also inhibited by approximately 80% in the caveolin-1 siRNA-transfected cells compared to untreated cells and cells transfected with the control siRNA (Fig 3.2.1.3.B). These results suggest that caveolae were most likely the primary membrane microdomains involved in PACAP signaling leading to neurite outgrowth of PC12 cells, and that perturbation to caveolar integrity would retard this process. Consistent with these results, another lipid rafts/caveolae modulator saponin, which disrupts plasma membrane DRM

A.

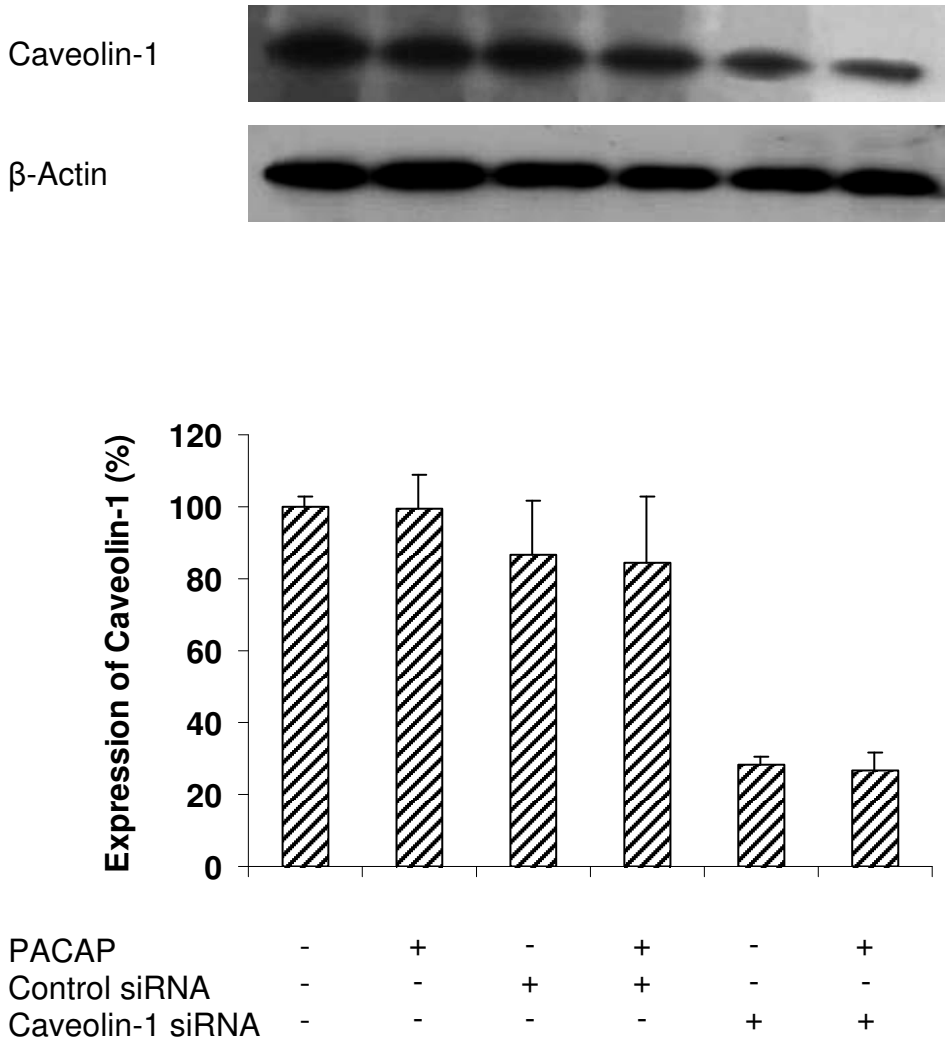


Fig 3.2.1.3. A. Effects of caveolin-1 siRNA on caveolin-1 expression in PC12 cells. Caveolin-1 expression in PC12 cells transfected with caveolin-1 siRNA or control siRNA for 48 h in the presence or absence of PACAP (100 nM) was detected and quantitated by Western blot analysis of the whole cell lysates. The blots were representatives of at least three independent experiments performed. β-Actin was used as the internal control. In the quantitative analysis, the mean expression levels of caveolin-1 (normalized against actin levels) in untreated PC12 cells was set as 100%. Values are mean ± SD of at least three independent experiments.

B.

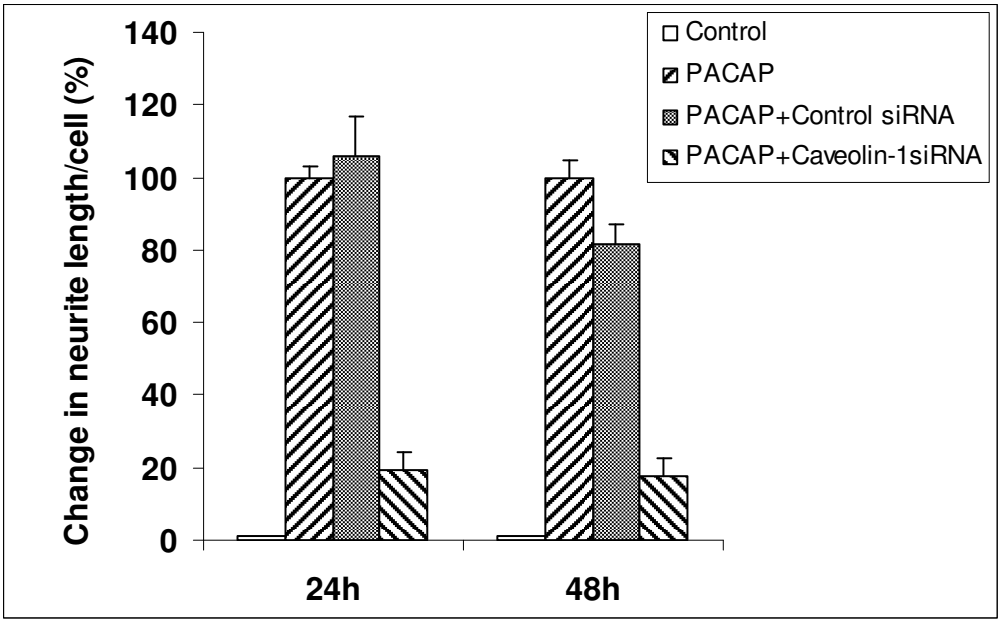


Fig 3.2.1.3. B. Effects of the disruption of the integrity of lipid rafts/caveolae on the PACAP-induced neurite outgrowth in PC12 cells. 24 h after seeding, PC12 cells were transfected with or without control siRNA or caveolin-1 siRNA. The transfection medium was replenished with fresh RPMI1640 medium containing 1% horse serum and PACAP 24 h after transfection. Phase contrast micrographs were taken at the time points of 24 h and 48 h after PACAP was added and neurite length/cell was quantified as described. The relative change was expressed as a percentage of the mean neurite length/cell for the cells treated with PACAP alone (100%). The results are shown as means ± SD for at least three independent experiments.

structures and alters their detergent-sensitive (Wassler et al. 1987; Montixi et al. 1998), also eliminated the PACAP-induced neurite outgrowth (data not shown), further confirming that the integrity of lipid rafts/caveolae was critical for this neurite extension process.

3.2.2. PACAP-induced neurite outgrowth inhibited by U18666a can be restored by exogenous cholesterol

To further investigate the function of raft lipids/caveolae cholesterol in neurite extension process of PC12 cells, cholesterol-methyl-β-cyclodextrin complex, which was used as an exogenous source of cholesterol, was applied to the PC12-stimulated cells with or without U18666a (Fig 3.2.2). Obviously, PACAP-induced neurite extension was potently enhanced by the added cholesterol complex (Fig 3.2.2). In the presence of PACAP, the neurite length per cell in cholesterol-treated cells was approximately two-fold longer than that in cells treated with PACAP alone (Fig 3.2.2), whilst in the absence of PACAP, the cholesterol complex on its own did not elicit neurite generation (data not shown). In addition, this cholesterol complex restored the U18666a-inhibited neurite extension to at least the level of cells treated with PACAP alone. Although the stimulatory effect of cholesterol in NGF-induced neurite outgrowth in PC12 cells was not as potent as that in PACAP-induced cells, the overall pattern of the effect of cholesterol on neurite extension was similar in both NGF- and PACAP-treated cells (data not shown). These data, together with the

previous results shown in Figs 3.2.1.1 and 3.2.1.2, suggest that modulating plasma membrane cholesterol levels affected neurite extension stimulated by both PACAP and NGF in PC12 cells. This is consistent with the notion that neurite extension was largely dependent on the proper function of lipid rafts/caveolae.

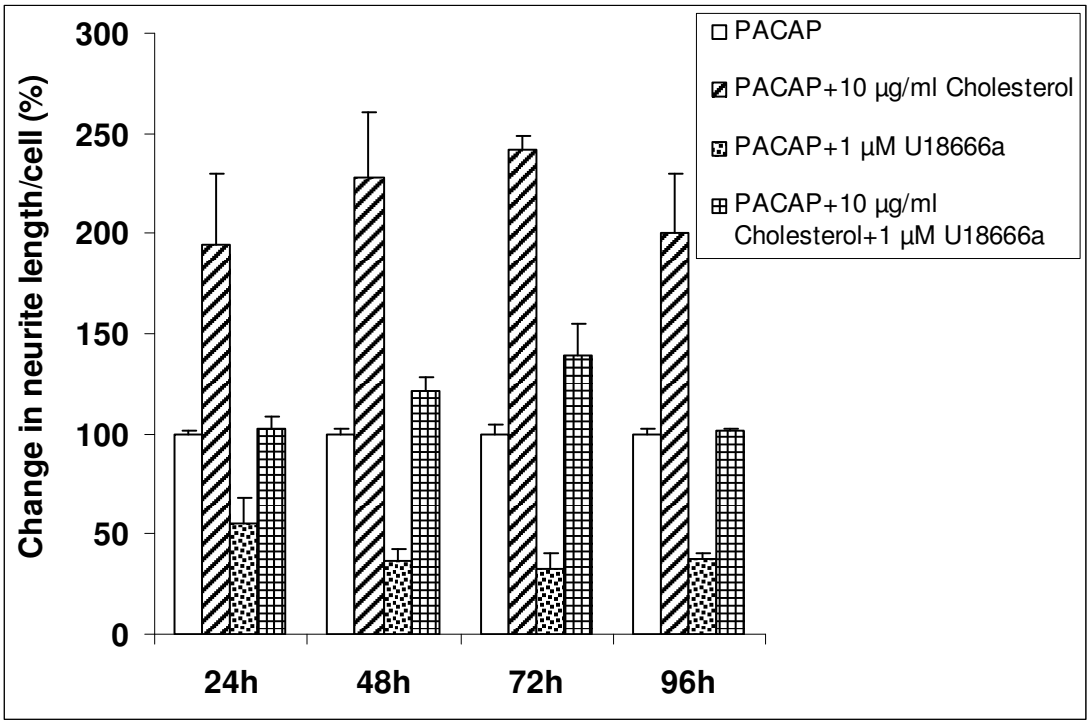


Fig 3.2.2. The dose-dependent effect of cholesterol on PACAP-induced neurite outgrowth and its effects on the U18666a-inhibited neurite outgrowth in PC12 cells. PC12 cells were cultured with 10 µg/ml cholesterol-CD (Cholesterol) or 1 µM U18666a or a combination of both in the fresh culture medium for 24 h. The average neurite length/cell was measured at time points of 24 h, 48 h, 72 h and 96 h after PACAP was added. The relative change was expressed as a percentage of the mean neurite length/cell for the cells treated with PACAP alone (100%). The results are shown as means ± SD for at least three independent experiments.

3.2.3. The neurite outgrowth inhibited by NB-DNJ can be restored by exogenous GM1

Our previous data show that inhibition to the biosynthesis of the lipid rafts/caveolae component glycosphingolipids by NB-DNJ retarded PC12 cells differentiation induced by PACAP (Fig 3.2.1.1), which indicate that sufficient level of glycosphingolipids was an essential cellular requirement for PC12 differentiation. To further verify this, PC12 cells were treated with NB-DNJ, or GM1, or both at different concentrations in the presence of PACAP. The phase contrast micrographs were taken for the following 4 days at indicated time points. Exogenous GM1, although on its own did not evoke neurite generation in the absence of PACAP (data not shown), potently promoted PACAP-induced neurite extension at different concentrations (except at a very high dose of 200 μM) (Fig 3.2.3). Interestingly, cell differentiation inhibited by NB-DNJ could be restored in the presence of GM1 in a concentration-dependent manner. Lower concentration of GM1 (1 μM) appeared to enhance neurite outgrowth more potently than higher GM1 concentrations (10, 100 μM) at early hours of treatment. However, only higher GM1 concentrations (10 or 100 μM) appeared to be able to sustain the neuite outgrowth. These results demonstrate that the effects of GM1 on neurite outgrowth in PACAP-treated PC12 cells were concentration-dependent. In addition, there appears to be an optimal range of GM1 concentration within which the signaling leading to neurite outgrowth could

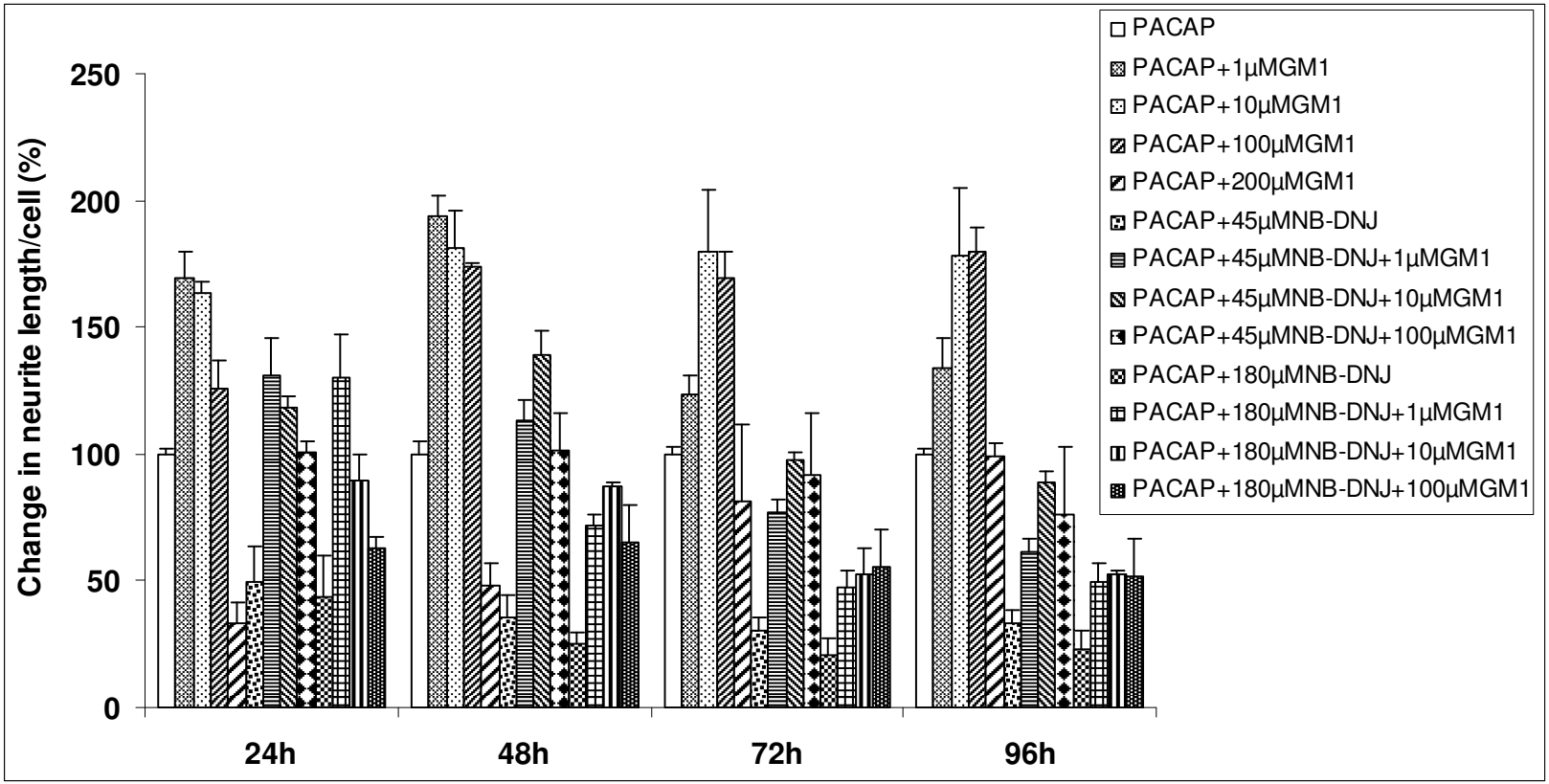


Fig 3.2.3. The dose-dependent effect of GM1 on the PACAP-induced neurite outgrowth and the restoration effects of GM1 on the NB-DNJ-inhibited neurite outgrowth in PC12 cells. PC12 cells pretreated with GM1 at various concentrations (1, 10, 100, 200 μM) or NB-DNJ (45, 180 μM) or combination of both for 24 h. Neurite length/cell was measured at time points of 24 h, 48 h, 72 h and 96 h after PACAP was added. The relative change was expressed as a percentage of the mean neurite length/cell for the cells treated with PACAP alone (100%). The results are shown as means ± SD for at least three independent experiments.

be enhanced, as overloading the cells with GM1 might result in retardation of neurite extension. The latter could be at least partly due to GM1-caused decrease in membrane fluidity (Nishio et al. 2004). Taken together, the results obtained above indicate that the inhibitory effects on the neurite outgrowth caused by depletion of lipid components of membrane rafts could therefore be reversed by exogenous supplement of these elements.

3.2.4. Disruption of the integrity of the lipid rafts/caveolae by caveolin-1 siRNA abolishes the enhancing effect of GM1 or cholesterol on the PACAP-induced neurite outgrowth

Previous data from this study show that disruption of the integrity of lipid rafts/caveolae by knock-down of the major structure protein of caveolae, caveolin-1, significantly reduced the PACAP-induced neurite outgrowth (Fig 3.2.1.3.B). To examine the effect of glycosphingolipid GM1 and cholesterol on the neurite extension inhibited by caveolin-1 siRNA treatment, the caveolin-1 knock-down cells were concurrently incubated with GM1 or cholesterol. In contrast to those results obtained in cells treated with NB-DNJ or the cholesterol-sequestering agents, caveolin-1 siRNA blocked completely the enhancing effect of both GM1 and cholesterol on PACAP-induced neurite outgrowth (Fig 3.2.4). In other words, the neurite extension inhibited by knocking down caveolin-1 expression, which might in turn disrupt the integrity of the lipid rafts/caveolae, cannot be restored by GM1 or cholesterol. These

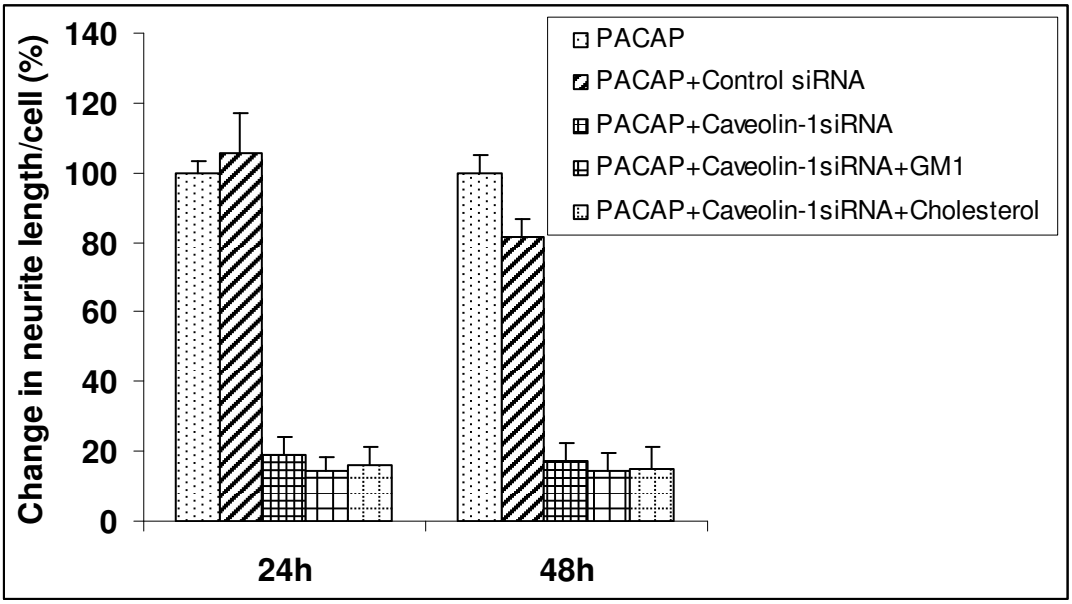


Fig 3.2.4. Effects of glycosphingolipids and cholesterol on the PACAP-induced neurite outgrowth in PC12 cells where the integrity of lipid rafts/caveolae were disrupted. PC12 cells were transfected with control siRNA or caveolin-1 siRNA in the presence or absence of GM1 (100 μM) or cholesterol (10 μg/ml) for 24 h. Phase contrast micrographs were taken at the time points of 24 h and 48 h after PACAP was added and neurite length/cell was quantified as described. The results are shown as means ± SD for at least three independent experiments.

results suggest that the presence of architectural and functional intact rafts/caveolae was critical for neurite extension.

3.2.5. Cholesterol level at the plasma membrane surface alters following treatment with exogenous cholesterol-methyl-β-cyclodextrin and cholesterol-depletion drugs

In the studies described above, we had assumed that plasma membrane cholesterol was depleted or replenished by the respective treatments. To verify whether cholesterol level was indeed altered at the plasma membrane surface following cholesterol-methyl-β-cyclodextrin or U18666a treatment, PC12 cells were treated with either of the above drugs for 24 h before performing filipin staining, a fluorescent polyene antibiotic widely used for the detection and quantitation of cholesterol in biomembranes. Fluorescence micrographs (Fig 3.2.5) show that addition of cholesterol-methyl-β-cyclodextrin complex clearly increased the membrane fluorescence intensity comparing to that observed from the untreated cells (Fig 3.2.5). On the other hand, fluorescence intensity on the plasma membrane was decreased in U18666a-treated cells, accompanied by an increase in intracellular accumulation of filipin staining as revealed by the appearance of spotty structures in the cytoplasmic fraction indicated by arrow bars (Fig 3.2.5). This would be expected since U18666a is an inhibitor of intracellular cholesterol trafficking (Liscum and Colliiins 1991; Sexton et al. 1983) and is widely used to mimic the Niemann Pick

Type C (NPC) disease cellular phenotype (Mohammadi et al. 2001; Pol et al. 2001; Runz et al. 2002; Liscum and Faust 1989; Hall et al. 2003), which is characterized by accumulation of intracellular cholesterol in lysosomes (Davies et al. 2000) resulting from defectiveness in cholesterol transportation to the plasma membrane (Wojtanik and Liscum 2003; Chang et al. 2005). These results therefore confirm that cholesterol-methyl-β-cyclodextrin and U18666a can either increase or decrease the plasma membrane cholesterol level respectively. Cholesterol enrichment in the cellular membrane surface boosted the PACAP-induced PC12 cells neurite extension and the effect was persistent within the time period of observation (Fig 3.2.2), whilst membrane cholesterol depletion restrained PACAP-induced neuritogenesis (Fig 3.2.1.1). These data were further confirmed by treatment with well-established cholesterol-sequestering drugs (Fig 3.2.1.2), such as filipin and nystatin, which sequester cholesterol within the lipid bilayer by inserting into plasma membrane (Simons and Toomre 2000), and methyl-β-cyclodextrin, which extracts cholesterol from plasma membrane (Klein et al., 1995; Schuck et al. 2003) and removes morphologically recognizable invaginated caveolae (Harder et al. 1997; Rodal et al. 1999). In addition, when supplemented with cholesterol after membrane cholesterol-depletion, neurite extension was resumed (Fig 3.2.2). These results show that plasma membrane cholesterol could be modulated *in vitro*. Clearly, alteration of plasma membrane cholesterol affected the extent of PACAP-induced neurite outgrowth in PC12 cells. Since cholesterol is one of the major components of the lipid rafts/caveolae, these data provide additional evidence that these plasma membrane

microdomains are very likely involved in the PACAP signaling pathways in PC12 cells.

3.2.6. GM1 level at the plasma membrane surface changes following treatment with exogenous GM1 and NB-DNJ

To ascertain that GM1 expression level was indeed altered at the plasma membrane surface following treatment with exogenous GM1 and NB-DNJ, PC12 cells were either treated with 45 μM NB-DNJ or exposed to 100 μM ganglioside GM1 or both for 24 h before staining with Cholera toxin B (CTxB)-Alexa 594, which specifically binds to the lipid rafts/caveolae-associated glycosphingolipids GM1. Both fluorescence micrographs (Fig 3.2.6.A) and flow cytometry profile (Fig 3.2.6.B) show marked changes in the cellular level of GM1 after the respective treatments. The addition of NB-DNJ clearly reduced the membrane CTxB-Alexa 594-conjugated fluorescence level to approximately 50% of that of the control cells, while GM1-treated cells had a dramatically increased intensity (Fig 3.2.6.C). When cells were treated with GM1 and NB-DNJ simultaneously, their effects were neutralized (Fig 3.2.6.C). The pattern of fluorescence intensity changes was consistent when observed with either fluorescene microscopy or flow cytometry. The results further confirm that GM1 and NB-DNJ either increased or decreased the plasma membrane glycosphingolipid GM1 levels effectively. Clearly, GM1 enrichment at the plasma membrane surface enhanced PACAP-induced PC12 cells neurite extension and the

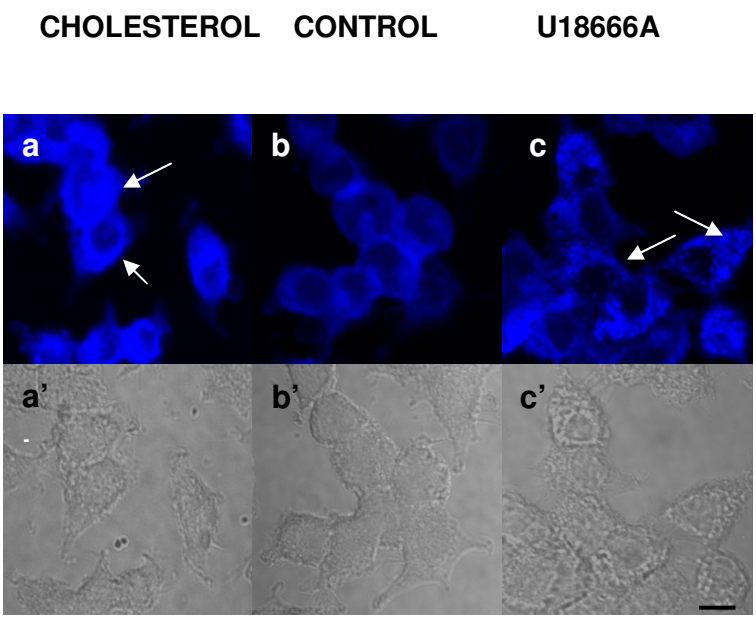


Fig 3.2.5. Relative expression levels of cholesterol in PC12 cells treated with exogeneous cholesterol-CD complex or U18666a. Fluorescence and corresponding phase contrast micrographs of control PC12 cells and those treated with cholesterol-CD (10 µg/ml, 24 h) or U18666a (1 µM, 24 h). a and a', cholesterol-CD treated PC12 cells; b and b', untreated PC12 cells; c and c', U18666a-treated PC12 cells. 50 µg/ml filipin was used to stain the cholesterol. The arrows in figure a indicate the increased cholesterol staining intensity comparing to the control cells. The arrows in figure c indicate the accumulated intracellular cholesterol staining. The scale bar represents 10 µm.

effect was sustainable within the time period of observation (Fig 3.2.4.A). GM1 depletion, on the other hand, suppressed the PACAP-induced neuritogenesis (Fig 3.2.1.1). Evidently, GM1 alone had no effects on neuritogenesis of PC12 cells in the absence of PACAP (data not shown). Hence, alteration of plasma membrane glycosphingolipid levels influenced neurite outgrowth induced by PACAP in PC12 cells. Since the glycosphingolipids on the plasma membrane are dominantly found in the lipid rafts/caveolae microdomains, these results further implicate a role for lipid rafts/caveolae microdomains in PACAP-induced neurite outgrowth of PC12 cells.

A.

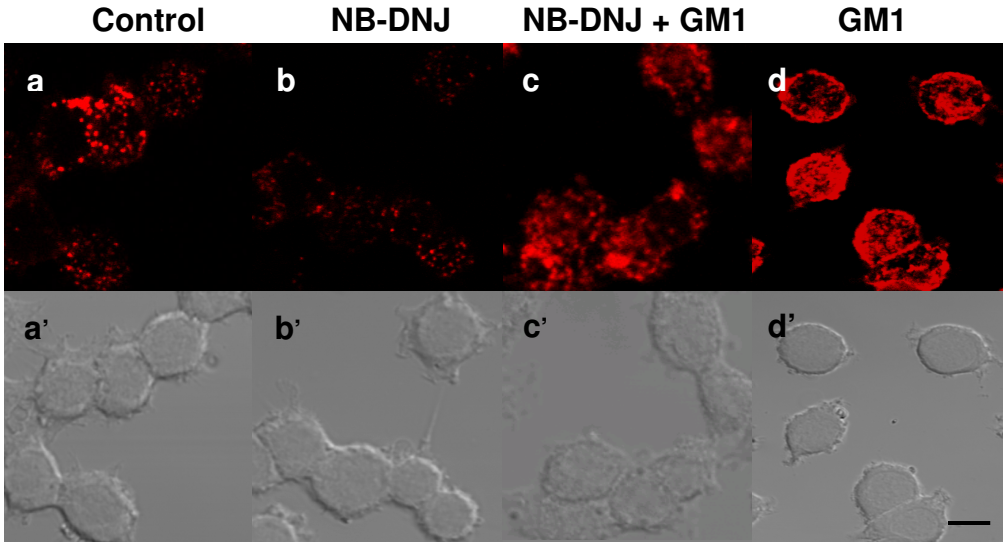


Fig 3.2.6. A. Relative expression levels of gangliosides GM1 in PC12 cells treated with exogeneous GM1 or NB-DNJ. Fluorescence and corresponding phase contrast micrographs of control PC12 cells and those treated with GM1 (100 µM, 24 h) or NB-DNJ (45 µM, 24 h) or both. a and a', untreated PC12 cells; b and b', NB-DNJ-treated cells; c and c', NB-DNJ and GM1-treated cells; d and d', GM1-treated cells. CTxB-Alexa 594 was used to stain cell surface GM1. The scale bar represents 10 µm.

B.

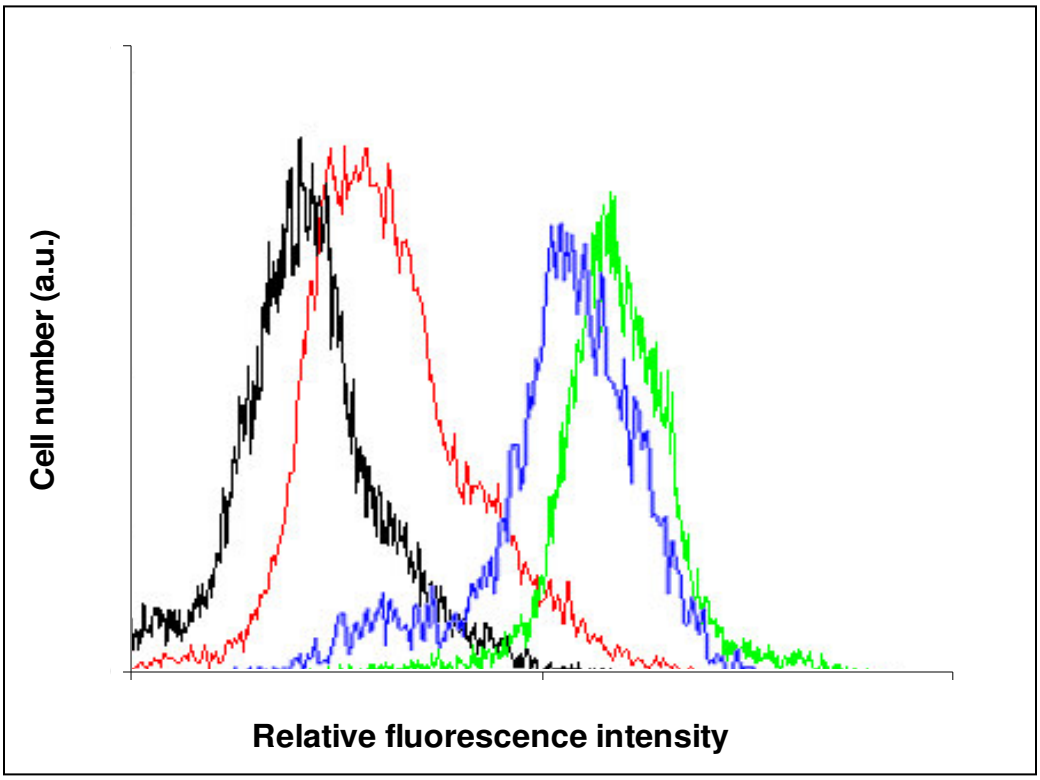


Fig 3.2.6. B. Relative expression levels of glycosphingolipid in PC12 cells treated with exogeneous GM1 or NB-DNJ. PC12 cells were treated as described in Fig 3.2.6.A, and stained with CTxB-Alexa 488 on ice for 30 min. The expression levels of cell surface GM1 was detected by flow cytometry. The profiles represent the untreated cells (in red), NB-DNJ-treated cells (in black), NB-DNJ and GM1-treated cells (in blue), and GM1-treated cells (in green).

C.

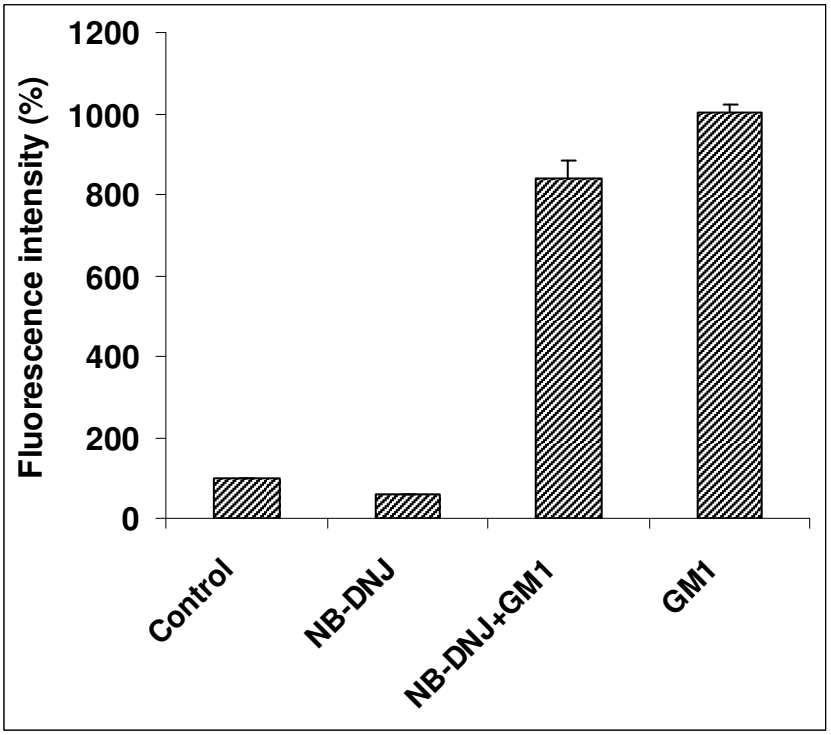


Fig 3.2.6. C. Quantitative analysis of CtxB fluorescence was based on Fig 3.2.6.B from three independent experiments.

CHAPTER 4. LIPID RAFTS/CAVEOLAE-MEDIATED PACAP SIGNALING PATHWAYS AND UNDERLYING MECHANISMS IN PC12 CELLS

4.1. Introduction

PACAP binds to PACAP-specific G-protein-coupled receptor family member to promote both neuronal differentiation and survival. In PC12 cells, addition of PACAP induces neurite outgrowth (as shown in Chapter 3) and inhibits cell proliferation (Deutsch and Sun, 1992; Hernandez et al. 1995; Lazarovici et al. 1998). In fact, PACAP-preferring receptor type I, PAC1R is the only endogenous G-protein-coupled receptor that could stimulate neurite outgrowth in PC12 cells (Tristan et al. 2003). In addition, PAC1R is expressed and positively coupled to adenylate cyclase (AC) 6, the type 6 isoform of adenylate cyclase found in PC12 cells (Deutsch and Sun 1992; Spengler et al. 1993; Oshikawa et al. 2003; Ravni et al. 2006). However, details of the molecular events leading to the coupling of ligand-bound PACAP receptors to downstream effectors are still unclear. In addition, the subsequent events leading to the eventual induction of neuritogenesis are also vague. The work described in this chapter focuses on the mechanistic role for the lipid rafts/caveolae in the regulation of PACAP-induced neurite elongation and their effects on the downstream signaling pathways.

4.2. Results and discussion

4.2.1. The expression level of PACAP receptor type I (PAC1R) is not adversely affected by perturbation of lipid rafts/caveolae

Since perturbation of caveolae altered PACAP-induced neurite outgrowth, the question was asked whether this was due to changes in the expression level of PACAP receptor type I: PAC1R. Exposure of PC12 cells to PACAP resulted in a marginal but statistically significant increase in PAC1R expression (Fig 4.2.1). However, treatments with nystatin, filipin, NB-DNJ or GM1, which would cause inhibition or promotion of PACAP-induced neurite outgrowth in PC12 cells, did not change the expression level of PAC1R any further compared to cells treated with PACAP alone. Moreover, PAC1R expression level was not affected by caveolin-1 siRNA. These data suggest that, although perturbation of caveolae might retard neurite outgrowth, it did not affect expression of PAC1R in PC12 cells.

4.2.2. PACAP induces partition of PAC1R into lipid rafts/caveolae microdomains and enhances its interaction with adenylate cyclase (AC)

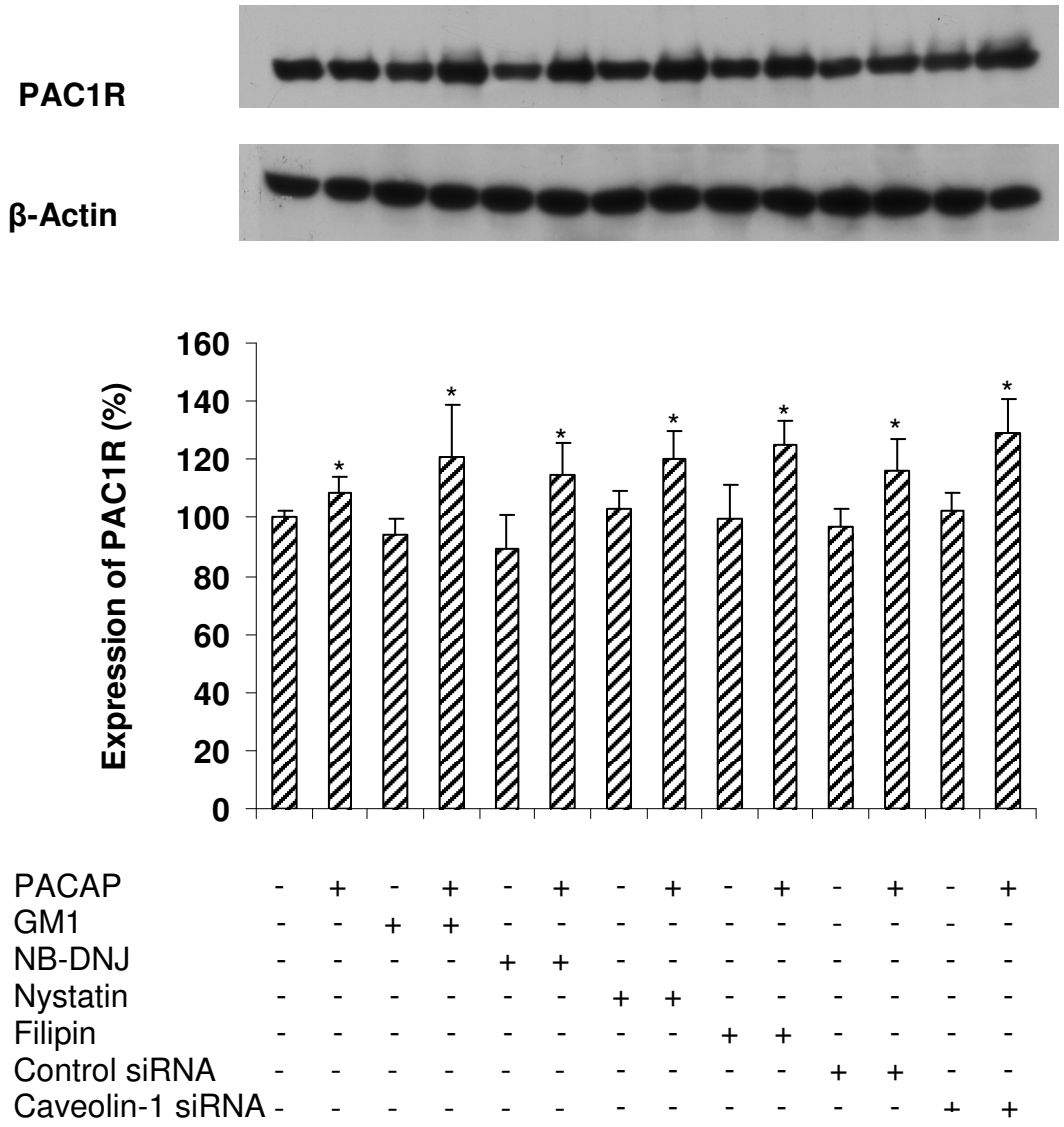
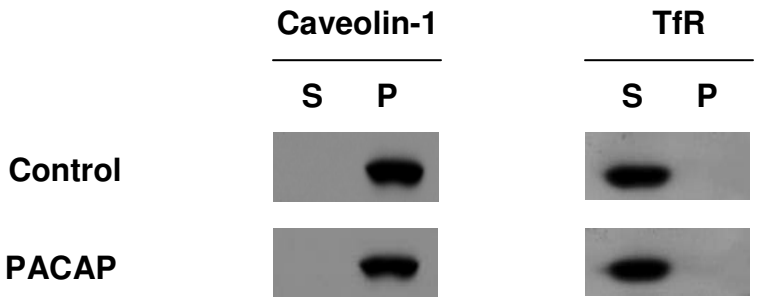


Fig 4.2.1. Expression of PAC1R in PC12 cells. Unstimulated or PACAP-stimulated PC12 cells were pretreated with various reagents as described in Materials and Methods. The whole cell lysates were used for immunobloting using anti-PAC1R antibody. The blots are representatives of at least three independent experiments performed. β-Actin was used as a loading control. In the quantitative analysis, the mean expression level of PAC1R in untreated PC12 cells was set as 100%. Values are mean ± SD of at least three independent experiments. *$p<0.05$ versus the unstimulated samples under identical treatment conditions.

To elucidate the molecular events following PACAP stimulation, the detergent solubility of PAC1R in unstimulated and PACAP-stimulated PC12 cells was investigated. As shown in Fig 4.2.2.A, the Triton X-100-insoluble fraction (P) was caveolin-1-enriched and free from the non-caveolar marker, transferrin receptor (TfR). Following PACAP stimulation, approximately 52 ± 4% of the total PAC1R were found in the detergent-insoluble fraction, a substantial increase compared to that found in unstimulated PC12 cells (37 ± 2%) (Figs 4.2.2.B and C). More PAC1R was translocated into caveolin-1-enriched detergent-insoluble fraction in GM1-loaded cells (75 ± 6%). In contrast, depletion of glycosphingolipids by NB-DNJ decreased the detergent insolubility of PAC1R to the level seen in unstimulated cells (39 ± 6%). Furthermore, treatments with filipin, nystatin and caveolin-1 siRNA, which were all expected to disrupt the structure of caveolae, prevented the translocation of PAC1R into the detergent-insoluble membrane microdomains (Figs 4.2.2.B and C). In addition, treatment of the cells with each of the above reagents in the absence of PACAP stimulation did not alter the distribution of PAC1R between the detergent-soluble and -insoluble fractions (data not shown), suggesting that PACAP stimulation is essential for the translocation of PAC1R to the detergent-insoluble membrane fraction.

The next question is whether altered membrane distribution of PAC1R facilitates its interaction with adenylnyl cyclase (AC), the target enzyme located in caveolae of various types of cells, including PC12 (Schwencke et al. 1999; Rybin et al. 2000;

A


Caveolin-1
TfR
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P
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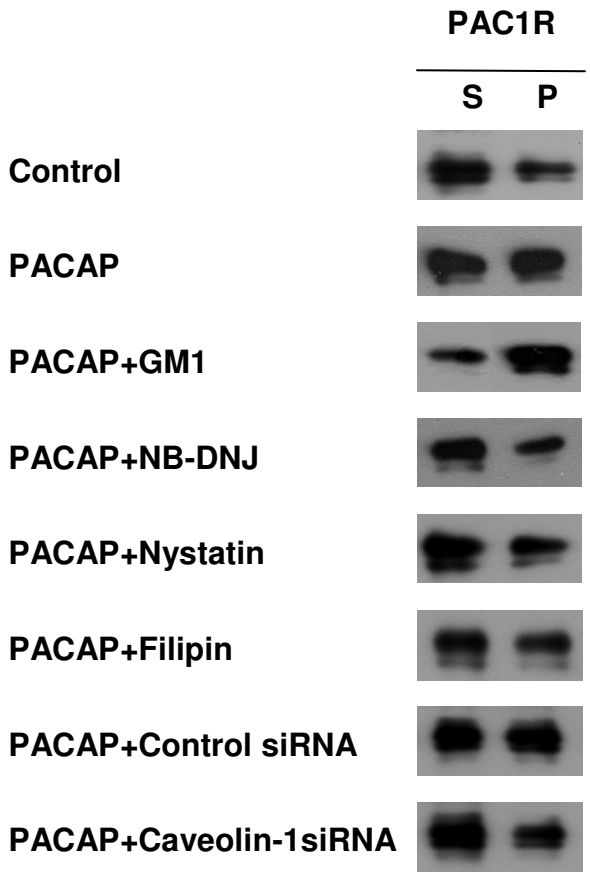
Control
PACAP

B


PAC1R
S
P
Control
PACAP
PACAP+GM1
PACAP+NB-DNJ
PACAP+Nystatin
PACAP+Filipin
PACAP+Control siRNA
PACAP+Caveolin-1siRNA

C.

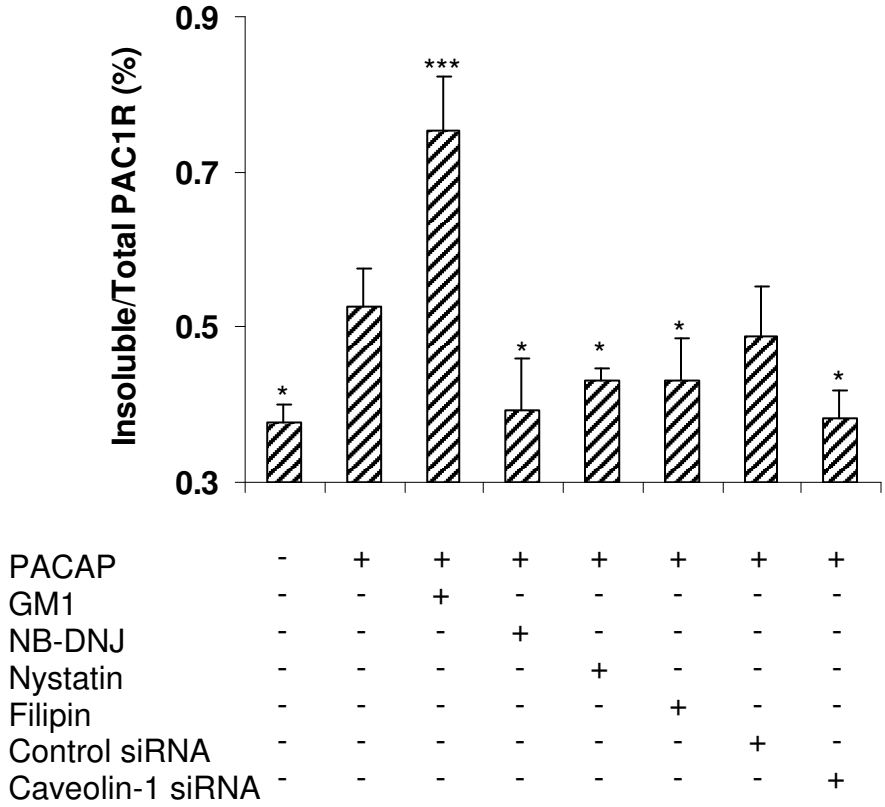


Fig 4.2.2. Translocation of PAC1R into caveolin-enriched detergent-insoluble fractions following PACAP stimulation. (A) PC12 cells were extracted with 1% Triton X-100 at 4 ^{o}C for 30 min and the soluble (S) and pelleted (P) fractions were separated by centrifugation. Presence of TfR and caveolin-1 in separate fractions was detected by Western blot with anti-TfR and anti-caveolin-1 antibodies, respectively. The blots are representatives of three independent experiments performed. **(B)** Identical aliquots of the detergent-insoluble and -soluble fractions obtained from PC12 cells treated under various conditions were processed by resuspending the pelleted fractions in the solubilization buffer and correcting the soluble fractions for the solubilization buffer components as described under "Materials and Methods". Immunoprecipitation was conducted with an anti-PAC1R antibody. The same antibody was used for the subsequent immunoblotting analysis. **(C)** Quantitative analysis on the relative change of the ratio of detergent-insoluble to total PAC1R for each of the treatment conditions. The results are shown as means ± SD for at least three independent experiments. *$p<0.05$ and ***$p<0.005$ versus the sample treated with PACAP alone.

Ostrom et al. 2000a; 2001; Oshikawa et al. 2003; Insel et al. 2005). Immunoprecipitation was performed with an anti-PAC1R antibody and the immunoprecipitate was probed with an anti-AC5/6 antibody (Fig 4.2.2.D). The level of AC associated with PAC1R in the PACAP-stimulated cells was approximately 4-fold higher than that in the untreated cells, suggesting much stronger interactions between PAC1R and AC, the effector enzyme that catalyzes the conversion of ATP to cAMP.

4.2.3. PACAP alters the distribution pattern of PAC1R in cell membranes

To further analyze whether the altered detergent solubility of PAC1R following PACAP stimulation was accompanied by changes in membrane distribution pattern of the receptor, immunostaining was performed for PAC1R and the staining pattern was visualized by confocal microscopy (Fig 4.2.3, middle column). PAC1R in the unstimulated cells showed a rather uniform plasma membrane distribution, whereas the receptors in the PACAP-stimulated PC12 cells showed a patchy distribution, indicating PACAP-induced accumulation of PAC1R in certain micro-regions of the plasma membrane. Addition of exogenous GM1 did not change the pattern of PAC1R distribution in the stimulated cells. However, depletion of GM1 by treatment of the cells with NB-DNJ caused dispersion of the patched PAC1R molecules. On the other hand, the distribution pattern of GM1 in the cell membrane was not altered by the

D.

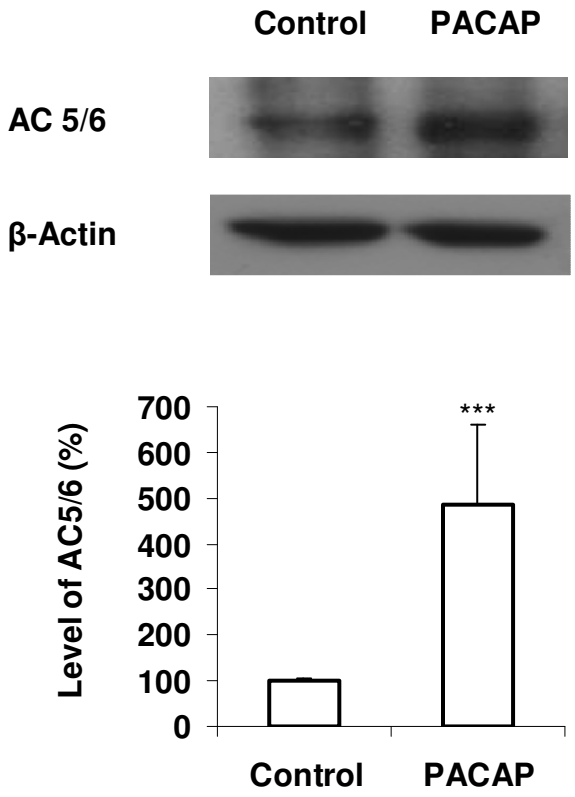


Fig 4.2.2. D. Increased affinity between PAC1R and AC after PACAP stimulation. Whole cell lysates obtained from untreated cells or cells stimulated with PACAP (100 nM, 5 min) were used for immunoprecipitation with an anti-PAC1R antibody, then for immunobloting using anti-AC5/6 antibody. β-Actin was used as a loading control. In the quantitative analysis, the mean level of AC in untreated PC12 cells was set as 100%. Values are mean ± SD of at least three independent experiments. ***$p<0.005$ versus the untreated controls.

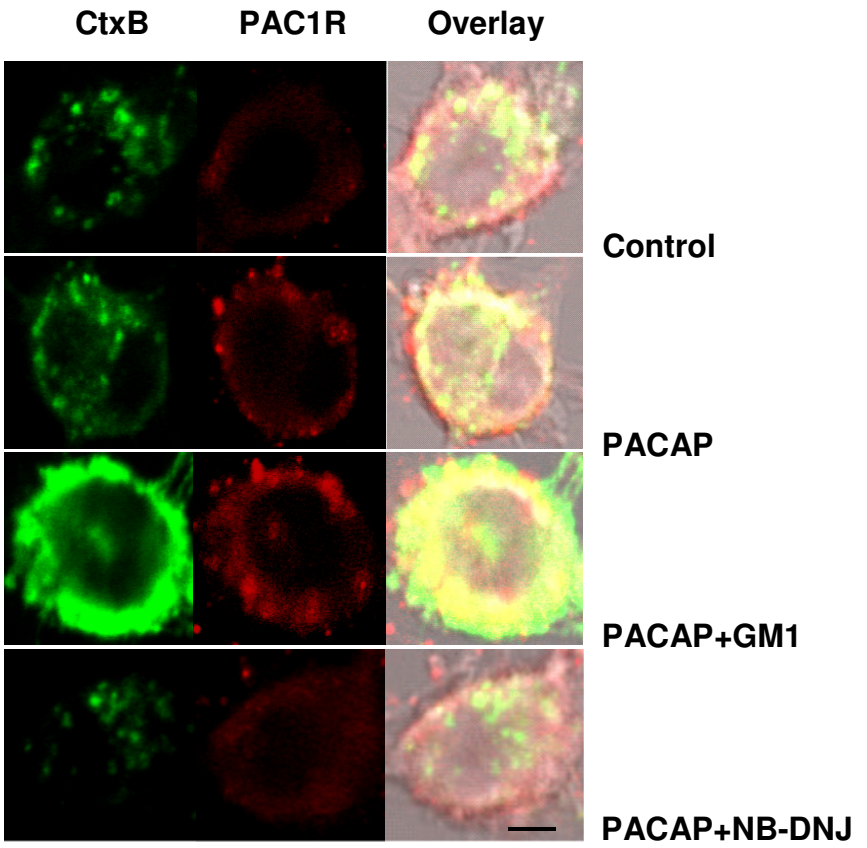


Fig 4.2.3. **Effect of PACAP stimulation on PAC1R distribution in cell membranes**. PC12 cells were cultured on glass coverslips and some of them were pretreated with GM1 (100 μM, 24 h) or NB-DNJ (45 μM, 24 h), respectively, followed by stimulation with PACAP (100 nM, 5 min), as indicated, before being fixed and stained with anti-PAC1R antibody and cultured in the presence of a TRITC-conjugated secondary antibody (red). The cells were then stained with CTxB-Alexa 488 (green). Note that the regions with a red-green overlap leads to yellow in the merged image (right column). The micrographs shown are representative of three independent experiments. Bar length represents 5 μm for all micrographs.

addition of PACAP, which showed a patched distribution in both the unstimulated control and the PACAP-stimulated cells (Fig 4.2.3, left column). Evidently, the level of colocalization between PAC1R and GM1, a lipid marker for the detergent-insoluble microdomains of cell membranes, was increased following PACAP stimulation, particularly in GM1-loaded cells (Fig 4.2.3, right column), in agreement with the results obtained by immunoprecipitation studies (Fig 4.2.2.B). Moreover, the distribution pattern of PAC1R was not affected by treatments with exogenous GM1 or NB-DNJ in the absence of PACAP (data not shown).

4.2.4. cAMP is involved in PACAP signaling and perturbation of caveolae affects intracellular cAMP synthesis

4.2.4.1. The effect of perturbation of caveolae on cAMP generation in PC12 cells

PAC1R is known to exert its major effect by coupling to AC, leading to the generation of cAMP. A prominent question to be asked is whether the cAMP synthesis stimulating ability of PAC1R was related to its translocation into caveolae. Fig 4.2.4.A shows that intracellular cAMP level was increased significantly by PACAP stimulation. When partition of PAC1R into caveolae was promoted by exposure of the cells to GM1, the cAMP level was elevated even further. In the

A.

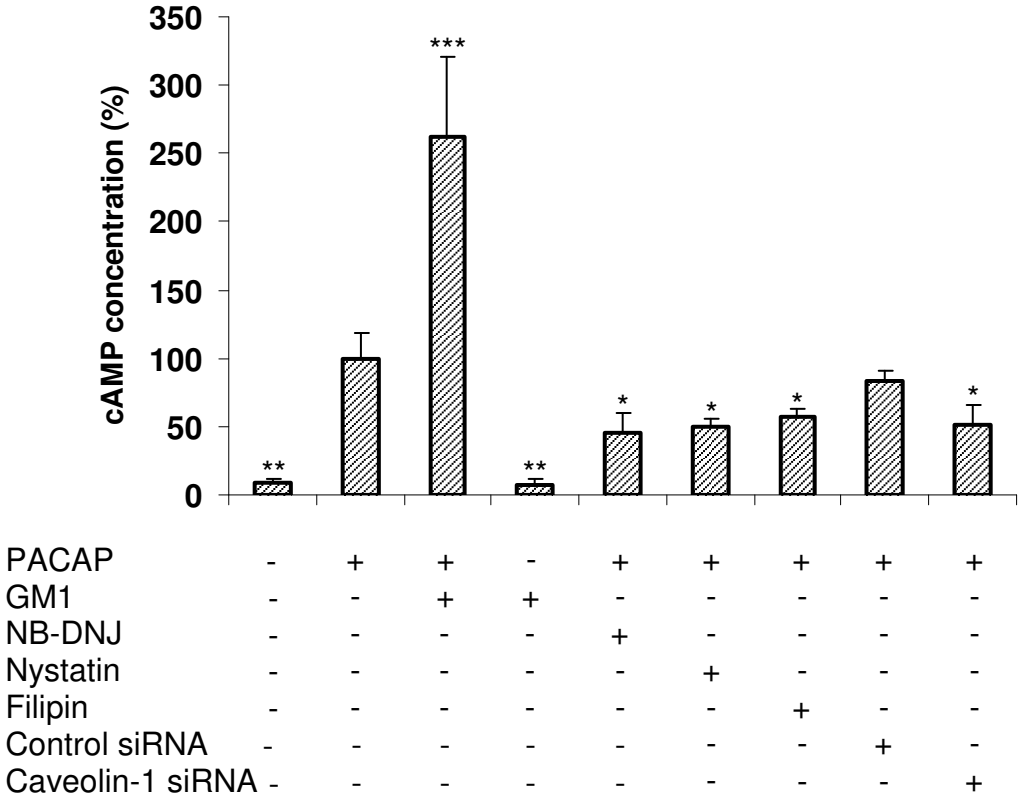


Fig 4.2.4. A. Effects of perturbation of caveolae on PACAP-stimulated cAMP generation and the critical role of cAMP signaling in neurite outgrowth of PC12 cells. Effects of various pretreatments (see "Materials and Methods" for details) on cAMP generation following PACAP stimulation (100 nM, 5 min). The results are shown as means ± SD for at least three independent experiments. *p<0.05, **p<0.01 and ***p<0.005 versus the samples treated with PACAP alone.

absence of PACAP, cAMP level was not increased by GM1 alone (Fig 4.2.4.A). In contrast, treatments which prevented PAC1R from translocating into the detergent-insoluble membrane microdomains resulted in a reduction in cAMP generation. Taken together, it appears that translocation of PAC1R into caveolae enhanced its coupling to AC and thus positively contributed to the regulation of intracellular cAMP production.

4.2.4.2. The involvement of cAMP in lipid rafts/caveolae-mediated PACAP signaling in PC12 cells

To test whether the cAMP pathway was directly involved in PACAP-induced neurite outgrowth of PC12 cells, cAMP signaling was specifically blocked by adding, prior to PACAP stimulation, RP-Adenosine 3', 5'-cyclic monophosphorothioate (RP-cAMP), a competitive membrane permeable cAMP antagonist, to PC12 cells. Fig 4.2.4.B shows that RP-cAMP significantly inhibited PACAP-induced neurite outgrowth at both concentrations used (0.5 mM and 1 mM). In addition, the enhancing effect of GM1 on the neurite extension was attenuated by RP-cAMP (Fig 4.2.4.B), reflecting the involvement of cAMP in rafts-mediated neurite elongation process elicited by PACAP.

On the other hand, adenosine 3', 5'-cyclic monophosphate, N^6, O^2-dibutyryl (db-cAMP), a cAMP agonist, could induce neurite outgrowth of PC12 cells in the absence

B.

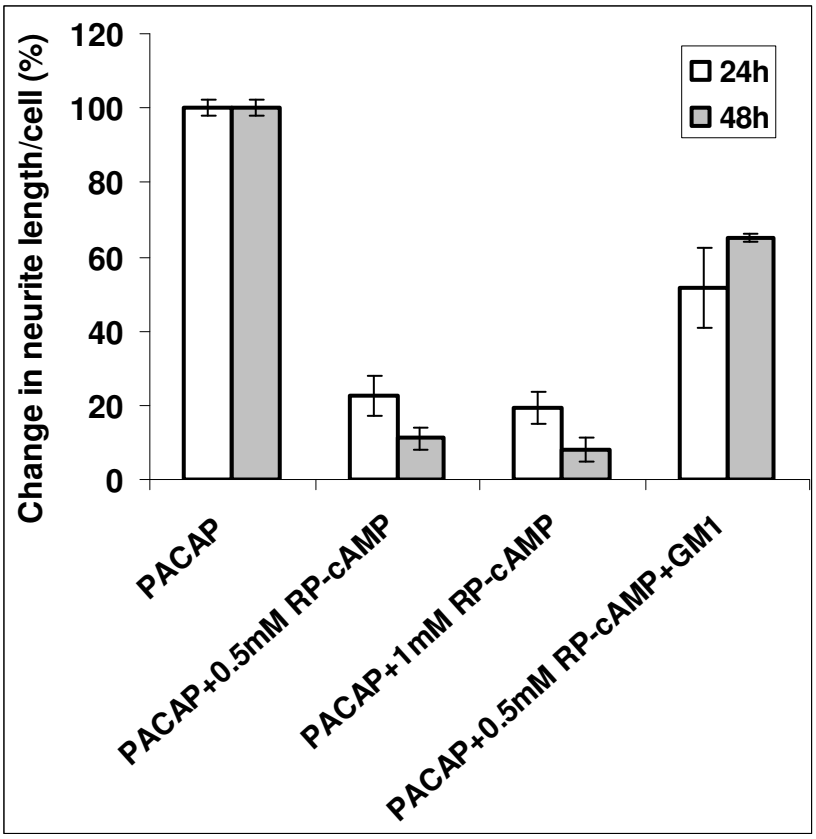


Fig 4.2.4. B. The inhibitory effect of RP-cAMP on the PACAP-induced neurite outgrowth and its effect on the function of glycosphingolipid in PACAP-induced neurite outgrowth. The mean neurite length/cell was measured at 24 h and 48 h in the presence of PACAP (100 nM), or a mixture of PACAP (100 nM) with RP-cAMP (0.5 mM or 1 mM) or a mixture of PACAP (100 nM), RP-cAMP (0.5 mM) and GM1 (100 μM) respectively, and the relative change was expressed as a percentage of the mean neurite length/cell for the cells treated with PACAP alone (100%), as described in Materials and Methods. Values are mean ± SD of at least three independent experiments.

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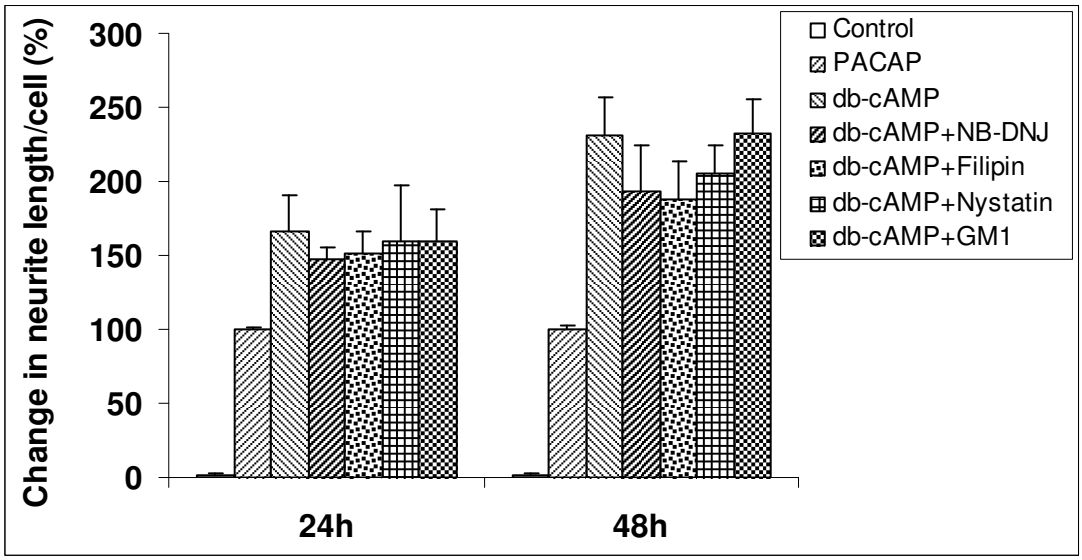

Fig 4.2.4. C. Effects of GM1 and the caveolar disruptors on PACAP analog, db-cAMP, caused neurite outgrowth of PC12 cells. The mean neurite length/cell was measured at 24 h and 48 h in the presence of PACAP (100 nM) or db-cAMP (1 mM). In some experiments, the cells were pretreated with NB-DNJ (45 μM) or GM1 (100 μM) or filipin (10 μg/ml) or nystatin (50 μg/ml). The relative change in neurite length was expressed as a percentage of the mean neurite length/cell for the cells treated with PACAP alone (100%), as described in Materials and Methods. The results are shown as mean ± SD for at least three independent experiments.

of PACAP, and this effect was even stronger than that by PACAP itself (Fig 4.2.4.C). These results and those of others (Gunning et al. 1981; Hernandez et al. 1995) suggest that cAMP signaling was necessary to induce neurite outgrowth in PC12 cells. Unlike PACAP, db-cAMP-induced neurite outgrowth in PC12 cells appeared not to be affected by perturbation of caveolae, as demonstrated by addition of exogenous GM1 and other caveolar disruptors (Fig 4.2.4.C). However, the effect of GM1 on PACAP-induced neurite extension was significantly attenuated by perturbation of caveolae (Fig 3.2.5.A), indicating that the amount of cAMP produced in caveolae, but not other possible secondary sources, was responsible for the effect of GM1. Therefore, these observations demonstrate that rafts/caveolae microdomains regulated the PACAP signaling through cAMP in PC12 cells.

4.2.5. ERK kinase1/2 (MEK1/2) is regulated by PACAP in PC12 cells

It has been reported that NGF-induced neuronal differentiation in PC12 cells is associated with extended activation of ERK (Qui and Green 1992), which has been shown to be necessary for neurite outgrowth (Fukuda et al. 1995). The molecular mechanisms involved in transducing signals from PACAP receptors to downstream targets such as ERK, however, are less defined than those for NGF. Here, PACAP-stimulated ERK phosphorylation and the regulation of its activation were investigated. The level of activated ERK was assessed using an anti-phospho-ERK antibody that

detects the dual Threonine (Thr)-202 / Tyrosine (Tyr)-204 phosphorylated forms of the kinase.

4.2.5.1. The sustained activation of ERK1/2 induced by PACAP

As expected, PACAP induced an increase in the levels of phosphorylation of ERK1/2 at Thr-202 and Tyr-204 (Fig 4.2.5.1.A). ERK1/2 phosphorylation reached a maximum level upon 5 min-stimulation with PACAP and gradually faded away but remained above the basal level (untreated cell) for a few hours (Fig 4.2.5.1.A). Compared to the phosphorylation level of ERK1/2 at 5 min post-stimulation with PACAP, it decreased by approximately 20% after incubation for 15 min and 50% for 30 min-incubation (Fig 4.2.5.1.C). On the other hand, NGF-induced ERK1/2 phosphorylation remained at a similar level after 15 min of stimulation compared to that of 5 min-incubation. It declined slightly (approximately 10%) after incubation for 30 min and remained approximately 70% of that observed at 5 min after stimulation for 60 min (Fig 4.2.5.1.C). The dynamic profile of the PACAP-evoked ERK1/2 phosphorylation was therefore similar to that for NGF (Fig 4.2.5.1.B). However, the effect of NGF on ERK1/2 activation was more pronounced and sustained. These observations were consistent with the more robust effect of NGF than that of PACAP on neurite outgrowth (data not shown).

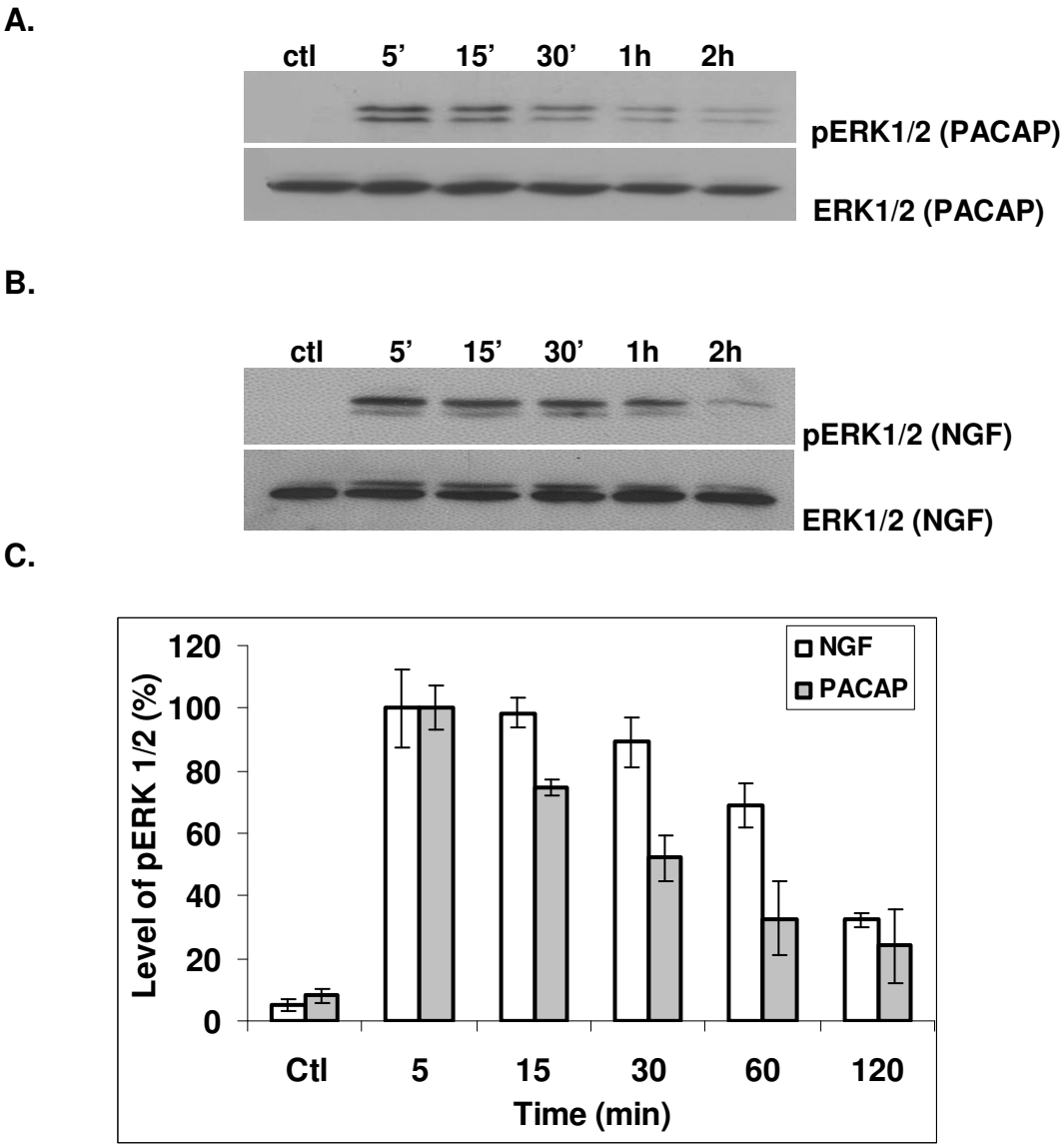

Fig 4.2.5.1. The phosphorylation level of ERK1/2 upon PACAP and NGF stimulation in PC12 cells. PC12 cells were incubated with or without (control) PACAP **(A)** or NGF **(B)** for the indicated time periods (5 min, 15 min, 30 min, 1 h or 2 h). The above treated cells were then harvested and subjected to SDS-PAGE and Western blotting using a phosphospecific ERK1/2 (pERK1/2) antibody. Equal loading was verified by probing the blots with an anti-ERK1/2 (ERK1/2) antibody. The data are representative of three independent experiments. **(C).** Quantitative analysis of the relative change of the level of phosphorylated ERK1/2 for each of the incubation time period with or without (control) the PACAP or NGF versus that of the cells treated for 5 min (100%) was obtained by densitometry determination. Values are mean ± SD of at least three independent experiments.

4.2.5.2. The essential role of MEK1/2 activation for PACAP-induced activation of ERK1/2 and for the function of glycosphingolipid on the neurite extension

To further confirm that the ERK1/2 phosphorylation was regulated upon PACAP stimulation and to examine the pertinent mechanism, the ERK kinase1/2 (MEK1/2)-specific inhibitor, PD98059, was used to treat the PC12 cells. PD98059 dramatically inhibited PACAP-induced phosphorylation of ERK1/2 from the early stage (5 min) of PACAP stimulation and this inhibitory effect was found to be sustainable (Fig 4.2.5.2.A). Moreover, morphological investigation of PC12 cells in response to PACAP treatment in the presence of PD98059 up to 48 h revealed that PD98059 abolished the ability of PACAP to induce neurite outgrowth in PC12 cells (Fig 4.2.5.2.B. middle column). In addition, PD98059 also obliterated the neurite growth stimulatory effect of glycoshingolipid GM1 (Fig 4.2.5.2.B, right column), suggesting that MEK1/2 is an essential downstream effector molecule in the process of lipid rafts/caveolae-modulated PC12 cells differentiation elicited by PACAP.

4.2.5.3. The effect of perturbation of lipid rafts/caveolae on ERK1/2 activation

Our experimental data showed that enrichment of the plasma membrane with either cholesterol or glycosphingolipid enhanced the PACAP-induced phosphorylation of

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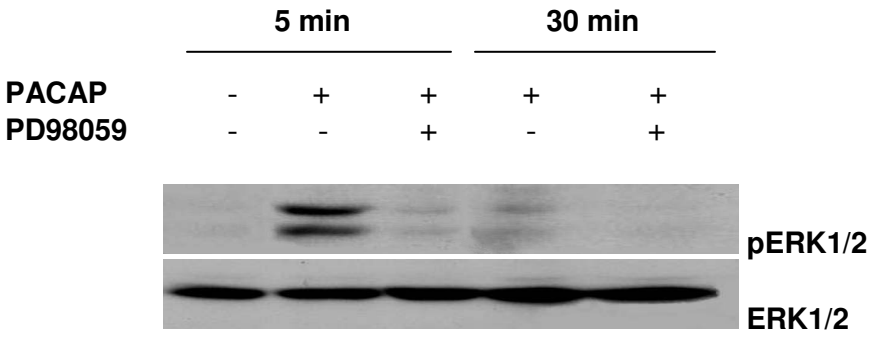


Fig 4.2.5.2. A. The inhibitory effect of PD98059 on the PACAP-induced phosphorylation of ERK1/2. PC12 cells were incubated with or without PD98059 (100 μM) for 1 h before incubated with PACAP for 5 min or 30 min. The samples were subjected to Western blotting using a phosphospecific ERK1/2 (pERK1/2) antibody. Equal loading was verified by probing the blot with an anti-ERK1/2 (ERK1/2) antibody.

B.

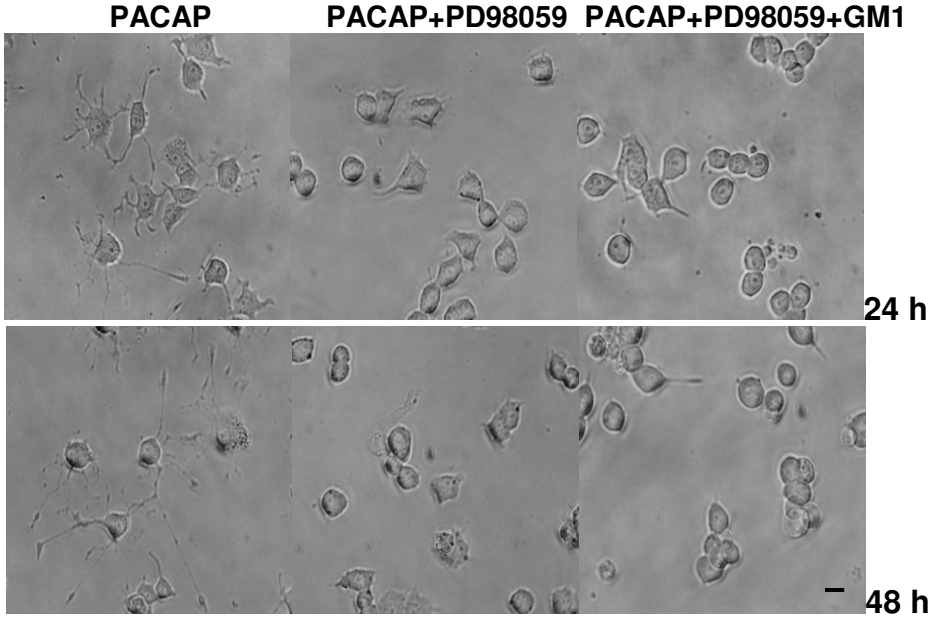


Fig 4.2.5.2. B. The effect of specific ERK inhibitor PD98059 on the function of glycosphingolipid and the PACAP-induced neurite outgrowth in PC12 cells. PC12 cells were cultured with RPMI1640 medium containing 1% horse serum and 100 nM PACAP in the absence or presence of either PD98059 alone or both PD98059 and 100 μM GM1. Phase contrast micrographs were taken after 24 h and 48 h after PACAP stimulation. The scale bar represents 10 μm.

ERK1/2, whilst depletion of either one of the rafts component attenuated the level of ERK1/2 phosphorylation (Fig 4.2.5.3 upper panel). These data were consistent with the finding that blocking ERK1/2 phosphorylation by MEK1/2 inhibitor abolished the morphological response of PC12 cells to PACAP (Fig 4.2.5.2.B, right column). Both suggest the critical role of ERK1/2 phosphorylation in PACAP-induced neurite outgrowth of PC12 cells. Taken together, these data demonstrate that perturbation of the lipid rafts/caveolae would inhibit PACAP-stimulated activation of ERK1/2 signaling cascade as well as subsequent neurite extension, while enhancements to ERK1/2 phosphorylation would promote the differentiation of PC12 cells (Figs 3.2.3 and 3.2.4).

4.2.5.4. The involvement of cAMP in the PACAP-stimulated activation of ERK1/2

To further investigate whether PACAP-induced ERK1/2 phosphorylation was regulated by cAMP, RP-cAMP, a specific antagonist of cAMP, was used to treat the cells (Fig 4.2.5.4.A). The activation of ERK1/2 was decreased by RP-cAMP (Figs 4.2.5.4.A and B), which inhibited the PACAP-induced neurite outgrowth as well (Fig 4.2.4.B). These data suggest that ERK1/2 phosphorylation was a downstream event of cAMP synthesis in PACAP-generated neurite extension pathway. On top of this, the stimulatory effect of GM1 and prohibitive effect of NB-DNJ on PACAP-induced ERK1/2 phosphorylation was simultaneously restrained in the presence of RP-cAMP

(Fig 4.2.5.4). These results indicate that plasma membrane rafts modulated PACAP-elicited ERK1/2 activation via cAMP, and that ERK1/2 was the effector protein involved in the downstream signaling of PACAP signaling pathways.

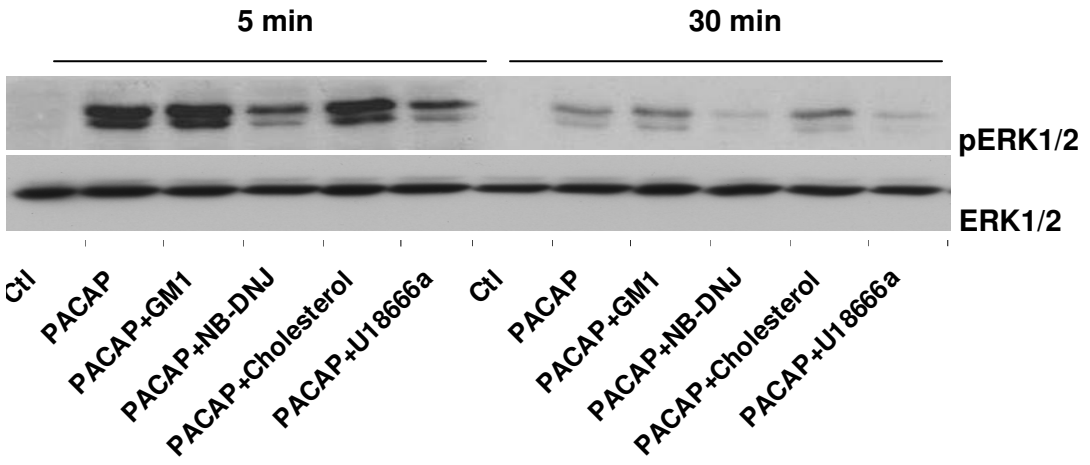


Fig 4.2.5.3. The effect of cholesterol and glycosphingolipids on PACAP-induced phosphorylation of ERK1/2. PC12 cells were treated with GM1, NB-DNJ, cholesterol-mβCD or U18666a, respectively, for 24 h before incubated with or without (control) PACAP for 5 min or 30 min, respectively. The cell lysates were subjected to SDS-PAGE and Western blotting analysis using a phosphospecific ERK1/2 (pERK1/2) antibody. Equal loading was verified by probing the blots with an anti-ERK1/2 (ERK1/2) antibody. The data are representative of three independent experiments.

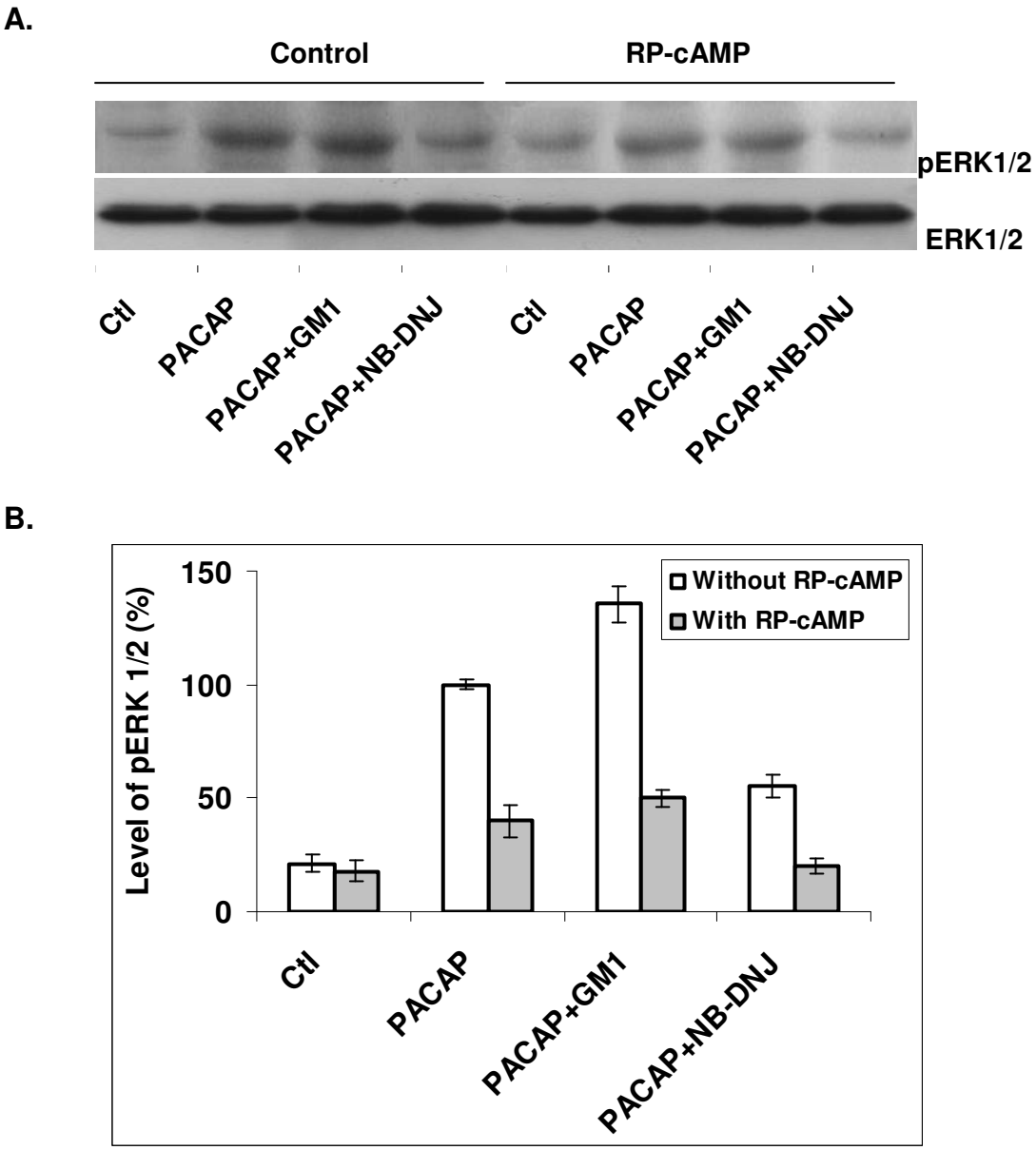


Fig 4.2.5.4. The inhibitory effect of RP-cAMP on the PACAP-induced phosphorylation of ERK1/2. (A). PC12 cells were treated with or without (control group) RP-cAMP. Both groups of cells were treated with GM1 or NB-DNJ for 24 h before incubation with or without (control) PACAP for 5 min. The samples were harvested and subjected to Western blotting using a phosphospecific ERK1/2 (pERK1/2) antibody. Equal loading was verified by probing the blot with an anti-ERK1/2 (ERK1/2) antibody. The data are representative of three independent experiments. **(B).** Quantitative analysis of the relative change of the level of phosphorylated ERK1/2 for each of the treatment with or without RP-cAMP versus that of the cells incubated with PACAP alone (100%) was determined by densitometry. Values are mean ± SD of at least three independent experiments.

4.2.6. PACAP signaling and the function of glycosphingolipid on PACAP-induced neuritogenesis are independent of protein kinase A activity

The most elaborately studied cAMP effector protein in mammalian cells was, until recently, the cAMP dependent protein kinase A (PKA), whose mechanisms of activation and structure have been investigated in detail. To determine whether the PACAP-stimulated neurite extension was attributed to activation of PKA, two pharmacological blockers of PKA, H89 and protein kinase inhibitor (PKI), were applied to treat the PC12 cells. H89 is a classical and potent PKA inhibitor, while PKI is a cell permeable selective PKA inhibitor which contains a pseudo-substrate sequence for PKA and inhibits PKA enzymatic activity by binding to free C subunits (Scott et al. 1985). Interestingly, neither of these inhibitors tested affected PACAP-induced neurite outgrowth in PC12 cells (Fig 4.2.6.A). In addition, the PACAP-stimulated ERK1/2 activation level was not influenced by the inhibition of PKA either at early stage (5 min), or the prolonged incubation (30 min) with PACAP (Fig 4.2.6.B). These data suggest that PKA might not be involved in PACAP-stimulated neurite extension. To further confirm this and test the specificity and efficiency of these pharmacological blockers, PKA activity was measured directly. As expected, the induction of PACAP did not affect the PKA activity statistically (Fig 4.2.6.C), which was consistent with the finding that inhibition of PKA did not influence PACAP-induced neurite extension (Fig 4.2.6.A). Both the inhibitors of PKA clearly

A.

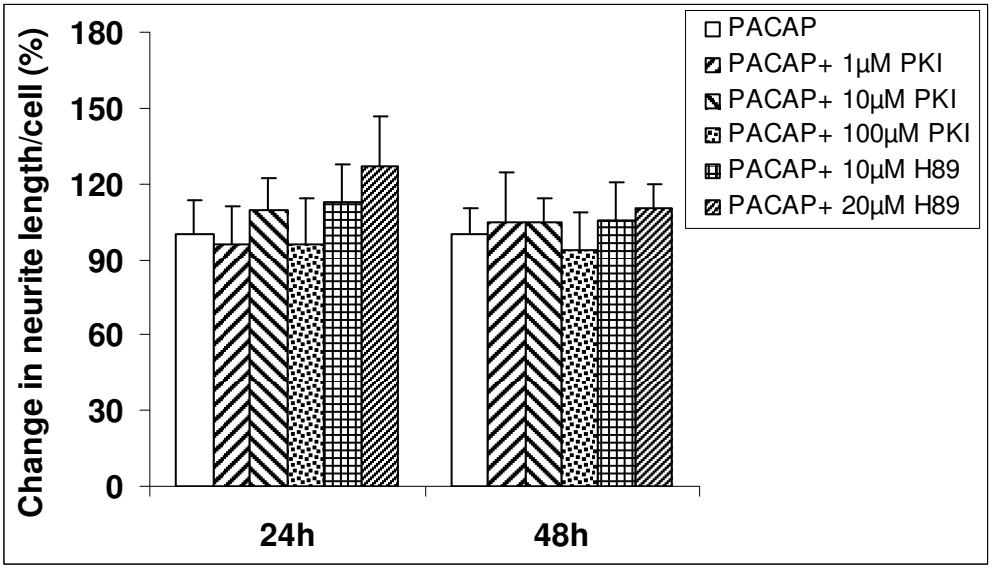


Fig 4.2.6. A. The effect of PKA on the PACAP-induced neurite outgrowth. PC12 cells were pretreated with different concentrations of cell permeable protein kinase inhibitor (PKI) (1, 10, 100 μM) or PKA inhibitor H89 (10, 20 μM) for 1 h before incubated with PACAP-containing medium. Neurite quantification analysis was measured at the indicated time points (24 h and 48 h). The relative change was expressed as a percentage of the mean neurite length/cell of the cells treated with PACAP alone (100%), as described in Materials and Methods. Values are mean ± SD of at least three independent experiments.

B.

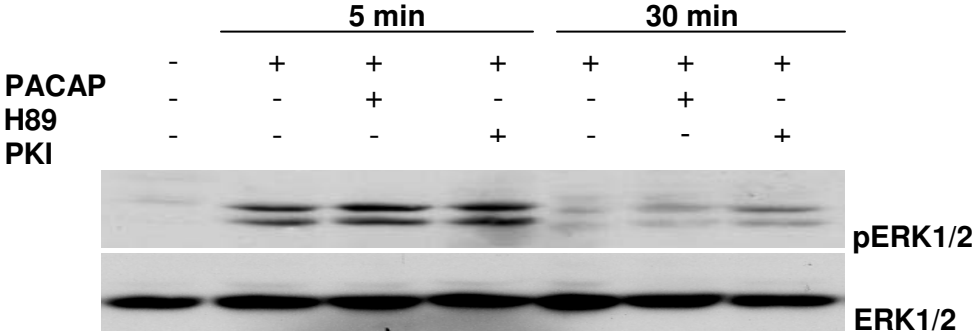
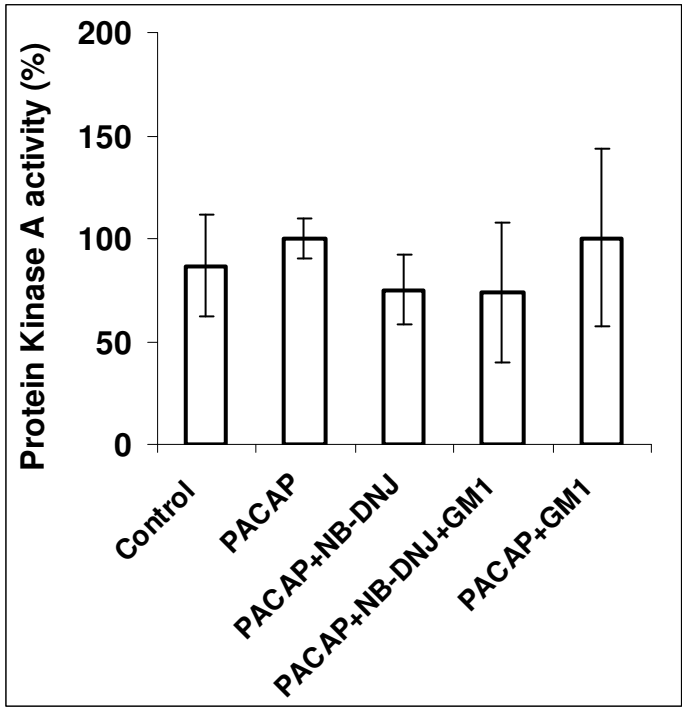


Fig 4.2.6. B. The effect of PKA on the PACAP-induced ERK1/2 activation. PC12 cells were pretreated with PKI (10 μM) or H89 (20 μM) for 1 h before incubated with or without PACAP containing medium for 5 min or 30 min. The samples were harvested and subjected to Western blotting using a phosphospecific ERK1/2 (pERK1/2) antibody. Equal loading was verified by probing the blots with an anti-ERK1/2 (ERK1/2) antibody. The data are representative of three independent experiments.

C.



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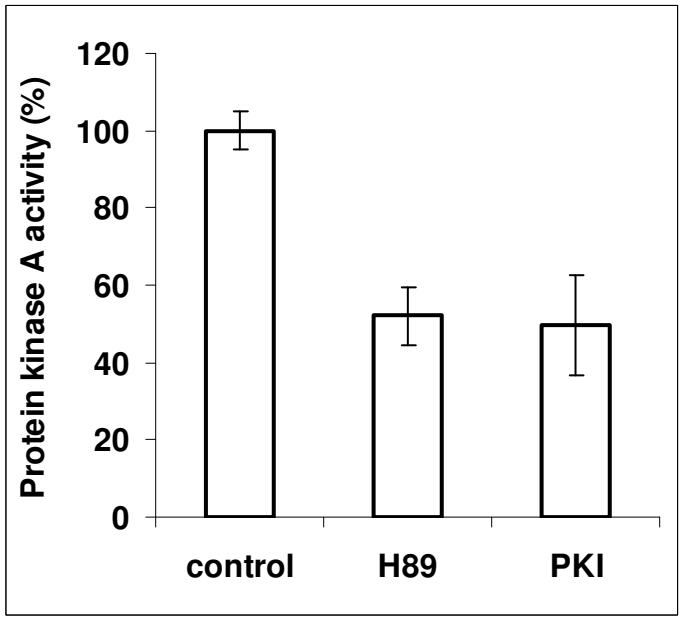

Fig 4.2.6. PKA activity assay. (C) PC12 cells were treated with NB-DNJ or GM1 or both for 24 h, or **(D)** treated with H89 (20 μM) or PKI (10 μM) for 1 h before harvesting for PKA activity assay, as described in the Materials and Methods. Values are mean ± SD of at least three independent experiments.

inhibited the PKA activity effectively (Fig 4.2.6.D). These results suggest that PKA activation was not necessary for PACAP-induced differentiation of PC12 cells. On top of this, the function of glycosphingolipid, the major component of membrane rafts/caveolae, either upregulated by GM1 or downregulated by NB-DNJ, on PACAP-induced neurite outgrowth was not affected by inhibition of PKA as well (Fig 4.2.6.E). On the other hand, PKA activity was not influenced by modulations of the membrane glycosphingolipid level with GM1 and NB-DNJ in PACAP signaling (Fig 4.2.6.C). Taken together, these results demonstrate that PKA activity was unlikely to be involved in the PACAP-stimulated neurite extension of PC12 cells, nor a signaling intermediate in rafts/caveolae-mediated PACAP signaling pathways.

4.2.7. The guanine nucleotide exchange factor (EPAC) is activated in the downstream of cAMP formation

It has long been questionable whether PKA is the only mediator of cAMP action (Dremier et al. 1997; Cass et al. 1999), until the discovery of **E**xchange **P**rotein directly **a**ctivated by **c**AMP (EPAC), a guanine nucleotide exchange factor for Rap1 (de Rooij et al. 1998; Kawasaki et al. 1998). EPAC is activated by cAMP through a cAMP-binding regulatory domain (Enserink et al. 2002). EPAC therefore provides an alternative path leading from PACAP-cAMP to MEK1/2 and ERK1/2 activation, independent of PKA.

E.

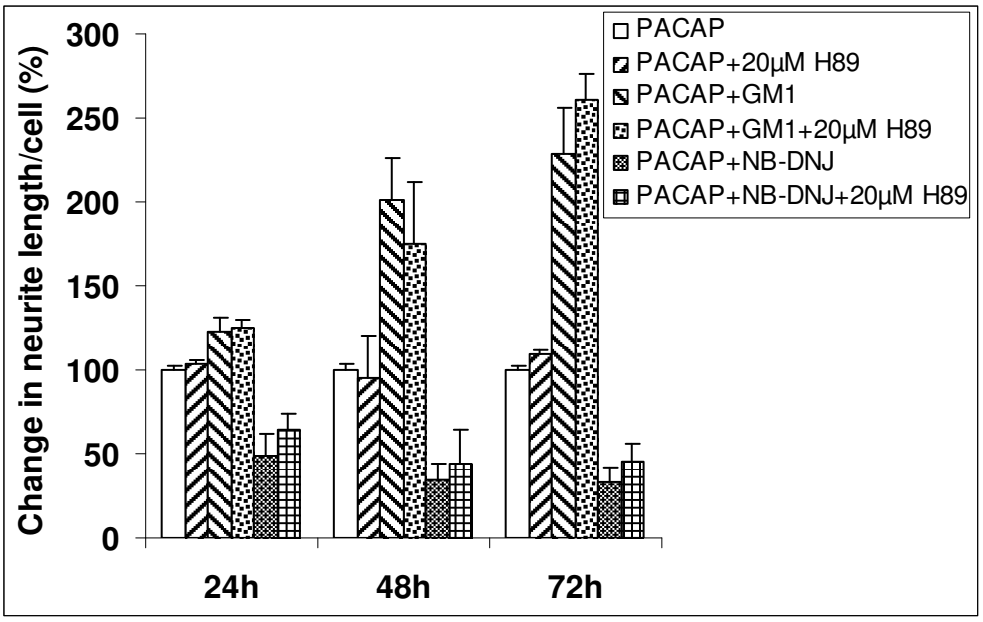

Fig 4.2.6. E. The effect of PKA on the PACAP-induced neurite outgrowth and the influence of membrane glycosphingolipid. PC12 cells were pretreated with or without GM1 or NB-DNJ for 24 h before incubated with H89 for 1 h. The medium was then changed to PACAP containing medium. The cells were cultured for another 72 h. The phase contrast photos were taken at the indicated time points (24 h, 48 h and 72 h). Neurite quantification analysis was carried out as described. The relative change was expressed as a percentage of the mean neurite length/cell of the cells treated with PACAP alone (100%), as described in Materials and Methods. Values are mean ± SD of at least three independent experiments.

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4.2.7.1. The effect of EPAC on neurite outgrowth in the absence or presence of PACAP and its effects on the function of membrane glycosphingolipid in PACAP signaling

The potential role of EPAC in PACAP-induced neurite outgrowth was tested next. The EPAC-selective activator, 8-cpt-2'-o-methyl-cAMP (8-OM-cAMP), an analog of cAMP that does not activate PKA (Christensen et al. 2003), provides a unique tool to activate EPAC without affecting other parallel signaling cascades. Fig 4.2.7.A shows that no stimulation of neurite outgrowth above background level was observed upon the addition of 8-OM-cAMP alone (Fig 4.2.7.A, column 1 and 2). Concurrent incubation with NB-DNJ (Fig 4.2.7.A, column 3) or GM1 (Fig 4.2.7.A, column 4) had no effect on neurite extension either. However, in the presence of PACAP, 8-OM-cAMP facilitated PACAP-induced neurite outgrowth and the average neurite length/cell was approximately 1.8-fold of that induced by PACAP alone during the first 24 h incubation (Fig 4.2.7.B). In addition, the inhibitory effect of NB-DNJ on PACAP-induced neurite elongation was rescued by the addition of 8-OM-cAMP (Fig 4.2.7.B). Taken together, these preliminary results indicate that EPAC was likely to be involved in the pathways of PACAP-stimulated neurite extension and its activity was affected by the integrity of lipid rafts/caveolae. However, EPAC activation on its own was insufficient to induce neurite extension, indicating that other pathway(s) activated by PACAP might collaborate with EPAC to induce neurite extension in PC12 cells.

4.2.7.2. The effect of EPAC on PACAP-stimulated activation of ERK1/2

To further establish the significance of EPAC in PACAP-stimulated activation of ERK1/2, the level of activated ERK1/2 was assessed in the presence or absence of PACAP (Fig 4.2.7.C). Treatment of PC12 cells with the EPAC activator 8-OM-cAMP alone did not increase the basal phosphorylation level of ERK1/2, which was consistent with the results of others (Enserink et al. 2002). On the other hand, in the presence of PACAP, EPAC activator robustly augmented MEK1/2-dependent ERK1/2 phosphorylation to a level approximately 4-fold higher than that induced by PACAP alone (Fig 4.2.7.C). This finding, coupled with the effects of EPAC activator on PACAP-elicited neuritogenesis (Fig 4.2.7.B), implicates a PACAP signaling cascade that EPAC could be involved in.

4.2.8. Both Rap1 and Ras are responsible for PACAP-induced and MEK1/2 -dependent ERK1/2 activation in PC12 cells

Ras and Rap1 are small GTP-binding G proteins of the Ras superfamily and govern a wide variety of cellular functions, including cell growth and differentiation (Ehrhardt et al. 2002; Quilliam et al. 2002). Guanine exchange factor (GEF) functions to promote the conversion of small G protein from a GDP-bound inactive form to a GTP-bound active form. On the other hand, GTPase-activating proteins (GAPs)

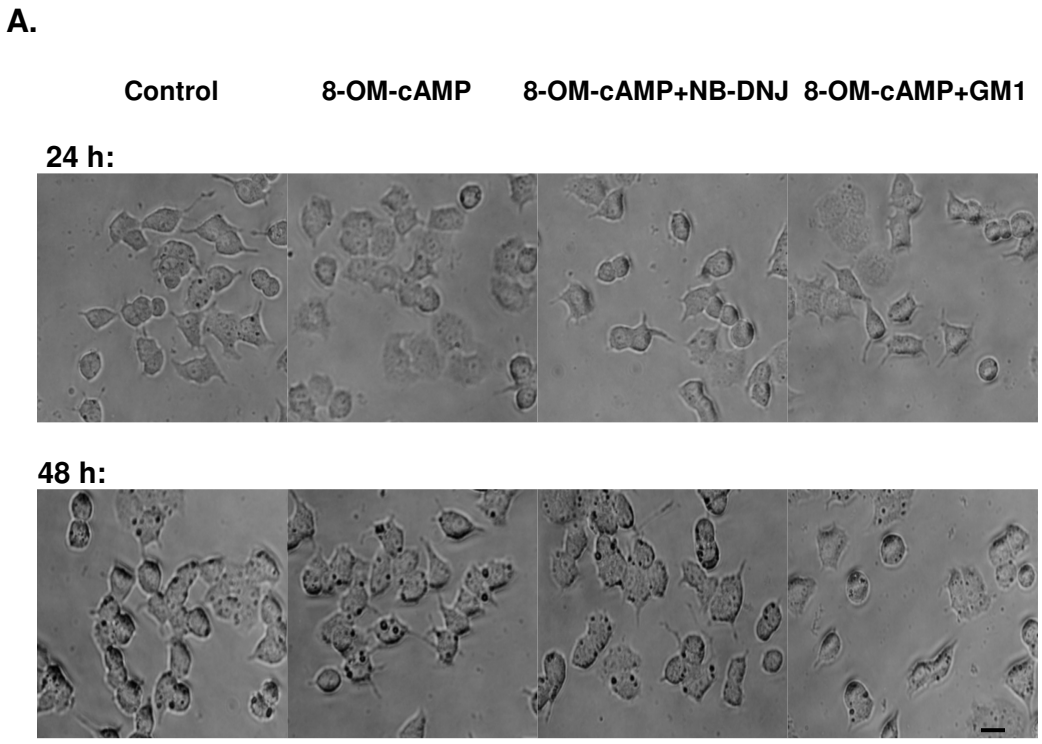

Fig 4.2.7. A. The effect of EPAC activator 8-OM-cAMP on the neurite outgrowth of PC12 cells in the absence of PACAP and the influence of membrane glycosphingolipid level in this process. PC12 cells were treated without (control) or with 8-(4-chlorophenylthio)-2'O-methyl-3', 5'-cyclic monophosphate (8-OM-cAMP) (1 mM) or a mixture of 8-OM-cAMP and NB-DNJ (45 µM) or GM1 (100 µM). Phase contrast micrographs were taken at 24 h and 48 h after treatment with 8-OM-cAMP. The scale bar represents 10 µm.

B.

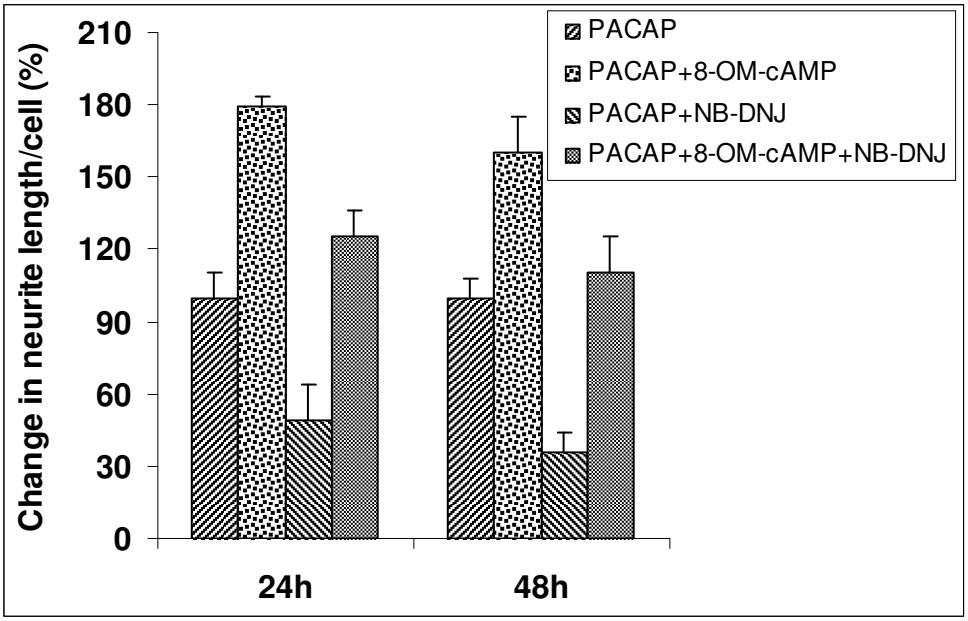

Fig 4.2.7. B. The effect of EPAC activator 8-OM-cAMP on the neurite outgrowth of PC12 cells in the presence of PACAP and the influence of membrane glycosphingolipid level in this process. PC12 cells were treated with or without 8-OM-cAMP (1 mM) in the presence or absence of NB-DNJ. The neurite length/cell was measured 24 h and 48 h after addition of PACAP. The relative change was expressed as a percentage of the mean neurite length/cell of the cells treated with PACAP alone (100%), as described in Materials and Methods. Values are mean ± SD of at least three independent experiments.

C.

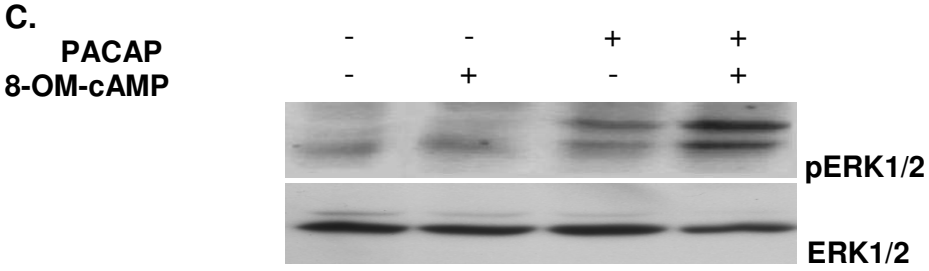

Fig 4.2.7. C. The effect of EPAC activator 8-OM-cAMP on PACAP-stimulated ERK1/2 activation. PC12 cells were pretreated with or without 8-OM-cAMP (1 mM) for 24 h before stimulation with PACAP for 5 min. The cells were then harvested and subjected to Western blotting using phosphospecific ERK1/2 (pERK1/2) antibody. Equal loading was verified by probing the blots with an anti-ERK1/2 (ERK1/2) antibody. The data are representative of three independent experiments.

promote the conversion from the active form to inactive form. Both Ras and Rap1 are targeted to membranes via their CAAX prenylation motifs (Ehrhardt et al. 2002; Hancock 2003), and their subcellular localization is also kinetically regulated (Maridonneau-Parini and de Gunzburg 1992). Rap1 and Ras have approximately 50% amino acid homology and share many binding partners, including ral guanine nucleotide dissociation stimulator (ral-GDS), phosphoinositol-3-kinase (PI3K), B-Raf and Raf-1 (Stork 2003).

4.2.8.1. PACAP-elicited sustained Rap1 activation and the critical role of Rap1 in the subsequent MEK1/2 -dependent ERK1/2 activation

Since EPAC is a Rap1 guanine nucleotide exchange factor that mediates Rap1 activation by direct binding to cAMP, Rap1 activation upon PACAP stimulation was examined. Fig 4.2.8.1.A shows that the activation, or GTP-loading, of Rap1 occurred very rapidly in the presence of PACAP and this activated form might sustain for a few hours. To establish that Rap1 is responsible for the PACAP-elicited activation of ERK1/2, a siRNA-based approach was used to reduce Rap1 protein levels. The efficacy of the inhibitory effect of Rap1 siRNA was evaluated concurrently with ERK1/2 activation assay. PC12 cells transfected with Rap1 siRNA led to a marked decrease (≈ 70%) in Rap1 expression compared to untransfected control cells (Fig 4.2.8.1.B, middle lower panel). Rap1-specific siRNA reduced the PACAP-stimulated ERK1/2 activation by near 40% from the very early stage of

A.

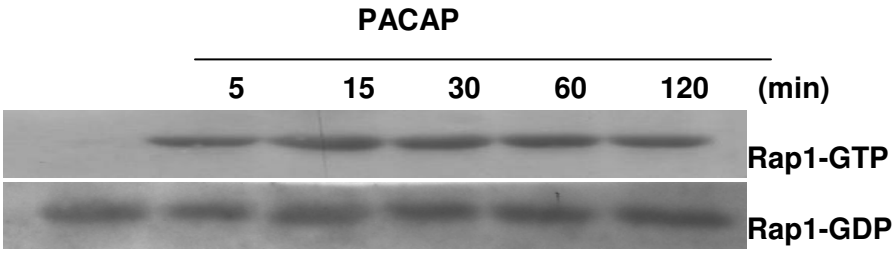

Fig 4.2.8.1. A. The Rap1 activation upon PACAP stimulation. PC12 cells were treated with PACAP for the indicated time periods and harvested. Rap1 activation assay was performed by Rap1 activation assay kit from Pierce and detected by anti-Rap1 antibody as described in Materials and Methods.

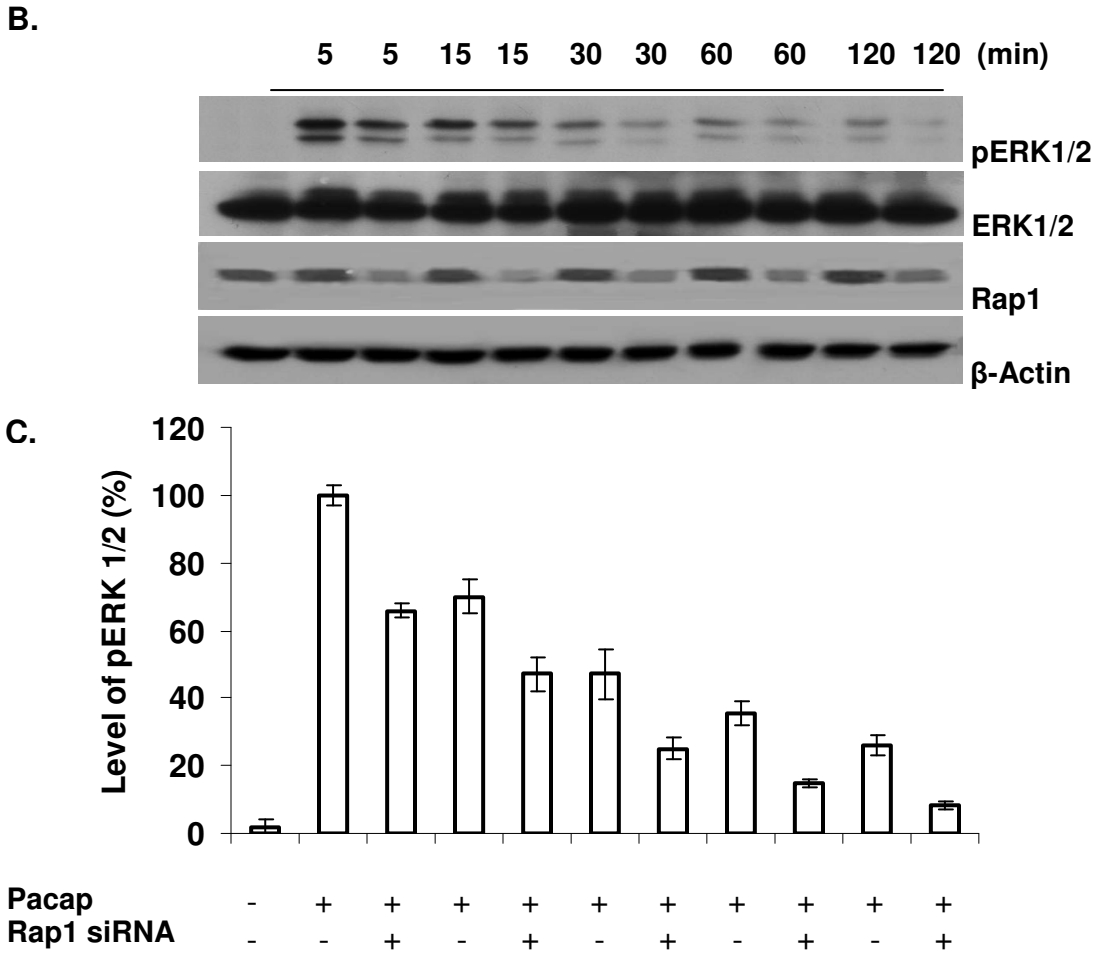

Fig 4.2.8.1. The effect of Rap1 on ERK1/2 activation. B. PC12 cells were transfected with or withoutwa Rap1 siRNA and then incubated with PACAP for the indicated time periods before harvested. The samples were subjected to Western blotting using Rap1 and phosphospecific ERK1/2 (pERK1/2) antibodies. Total ERK1/2 was shown by probing the blots with an anti-ERK1/2 (ERK1/2) antibody and equal loading was verified by probing the blots with an anti-β-actin antibody. The data are representative of three independent experiments. **C.** The relative decrease of pERK1/2 in Rap1 siRNA-treated cells versus that in the control PC12 cells at indicated time points was evaluated from the quantitative analysis of the Western blots from Fig 4.2.8.1. B. Values are mean ± SD of at least three independent experiments.

treatment in PC12 cells (Fig 4.2.8.1.B and C), whereas the total level of ERK1/2 in the cells was unaffected (Fig 4.2.8.1.B). This inhibitory effect could sustain for at least 2 h (Fig 4.2.8.1.B). Moreover, neurite length quantification shows that in Rap1-depleted cells, neurite extension was inhibited by approximately 50% comparing to the control siRNA-treated cells under similar experimental conditions (Fig 4.2.8.1.D). Taken together, these data suggest that, subsequent to EPAC activation, Rap1 played an important role in PACAP-elicited ERK1/2 phosphorylation, and was at least partially responsible for the PACAP-induced neurite elongation. On top of this, the glycosphingolipid GM1 could compensate the inhibitory effect of Rap1 siRNA on neuritogenesis, suggesting that the effect of rafts/caveolae on PACAP-induced neurite outgrowth might be somehow associated with Rap1 activation and this will be examined further in the next section.

4.2.8.2. The effect of perturbation of lipid rafts/caveolae on the activation of Rap1 and on the re-distribution of Rap1 between detergent-soluble and -insoluble membrane fractions

To further confirm that rap1 activation was associated with the function of lipid rafts/caveolae, caveolar disruptors were applied to the cell cultures. When plasma membrane was enriched with cholesterol or glycosphingolipid, the level of GTP-loaded Rap1 was increased (Fig 4.2.8.2.A). By contrast, depletion glycosphingolipid by NB-DNJ decreased PACAP-stimulated Rap1 activation level. Furthermore,

D.

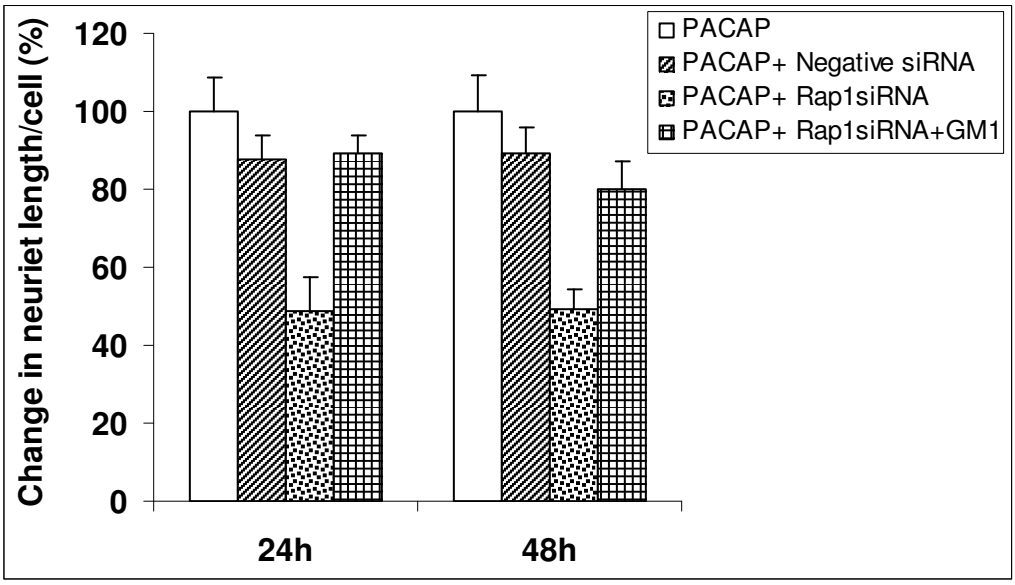

Fig 4.2.8.1. D. The effect of inhibition of Rap1 on the PACAP-induced neurite outgrowth and the influence of membrane glycosphingolipid level in this process. PC12 cells were transfected with control siRNA or Rap1 siRNA in the presence or absence of GM1 for 24 h. The neurite length/cell was measured at the indicated time points (24 h and 48 h, respectively) following PACAP stimulation. The relative change of the mean neurite length/cell was expressed as a percentage of that for the cells treated with PACAP alone (100%), as described in Materials and Methods. Values are mean ± SD of at least three independent experiments.

treatments with filipin, nystatin, methyl-β-cyclodextrin, and caveolin-1 siRNA, which were expected to disrupt the structure of lipid rafts/caveolae, all reduced Rap1 activation (Fig 4.2.8.2.A).

It has been reported that Rap1 becomes membrane-attached upon growth factor stimulation (Bivona et al. 2004; Li et al. 2005), and its subcellular localization is dynamically regulated (Maridonneau-Parini and de Gunzburg 1992). Therefore, it was of interest to check whether the distribution of Rap1 in the cell membranes was altered upon PACAP stimulation and upon perturbation of the lipid rafts/caveolae. As shown in Fig 4.2.8.2.B, cell lysate separated by a 10-40% continuous sucrose gradient was probed with anti-Rap1 antibody and anti-transferrin receptor (TfR) antibody. In untreated cells, the majority of Rap1 proteins were found in the first three light fractions which were free from the non-caveolar marker, TfR. There were some Rap1 found in the non-caveolar fractions (fractions 4-6) (Fig 4.2.8.2.B). Upon stimulation with PACAP, Rap1 in the non-caveolar fractions was largely diminished and a majority has moved to the caveolar fractions (fractions 1-3). In the presence of GM1, the extent of distribution of Rap1 to the caveolar fractions increased, leaving little Rap1 remained in the non-caveolar fractions. On the other hand, reduction of the plasma membrane glycosphingolipid by NB-DNJ dispersed the distribution of Rap1 to non-rafts fractions. In addition, when the cells were treated with other caveolar disruptors such as nystain, filipin, CD or caveolin-1 siRNA, Rap1 was also diverted to non-caveolar fractions, where less PAC1 receptor presented (shown earlier in Fig

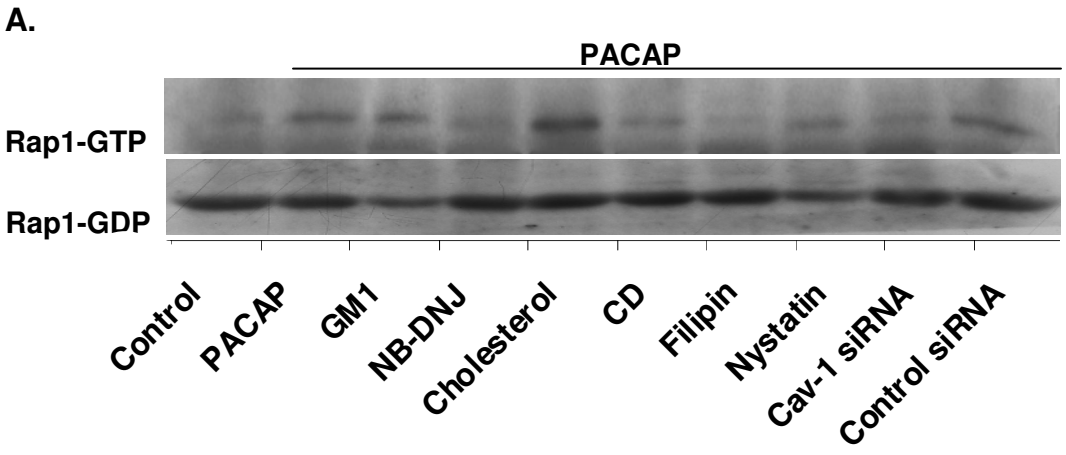

Fig 4.2.8.2. A. The effect of perturbation to the lipid rafts/caveolae on the activation of Rap1. PC12 cells were treated with GM1, cholesterol or the lipid rafts/caveolar disruptors before stimulated with PACAP for 5 min. The cells were harvested and performed Rap1 activation assay using the EZ-detect Rap1 activation assay kit from Pierce. In brief, activated GTP-loaded Rap1 protein was pulled down by GST affinity-precipitation using Rap1 binding domain GST-RalGDS-RBD and immunodetected with a Rap1 antibody. The GDP-bounded Rap1 protein was shown in the lower panel.

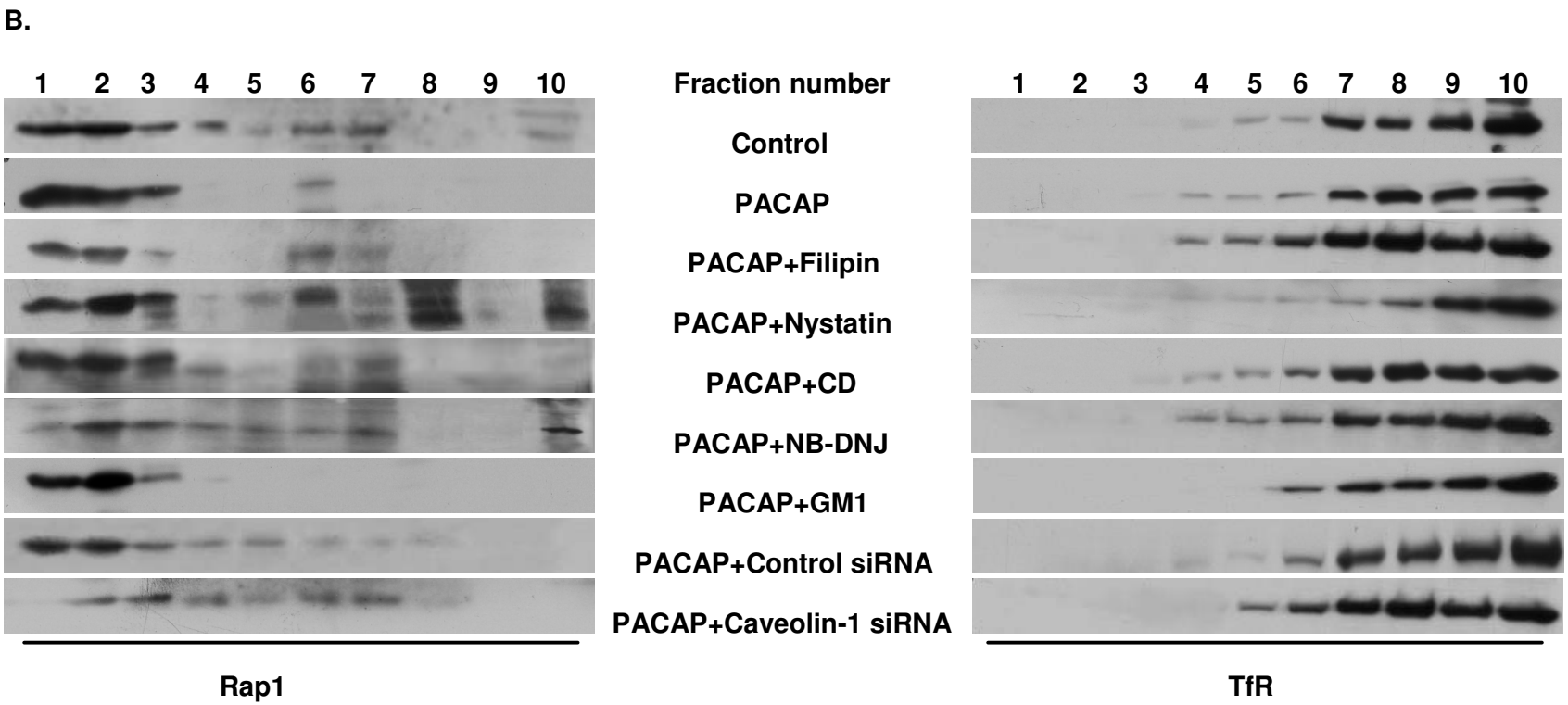

Fig 4.2.8.2. B. The effect of perturbation of lipid rafts/caveolae on the distribution of Rap1 in the membrane fractions. PC12 cells were treated with the above caveolae disruptors before PACAP stimulation for 5 min except the control. The cells were harvested and subjected to continuous sucrose gradient (10%-40%) separation as described in Materials and Methods. The fractions, whose density was in an ascending order from fraction 1 to 10, were probed with anti-Rap1 and transferrin-receptor (TfR) antibodies.

4.2.2.B). Taken together, these data demonstrate that upon stimulation with PACAP, Rap1 shifted to the caveolar fraction of the plasma membrane where signaling molecules were gathered, which was likely to facilitate the signaling cascade. Treatment of the cells with glycosphingolipid and cholesterol, which might stabilize and maintain the lipid rafts/caveolae microdomains, strengthened this translocation, and enhanced the assembly of the signaling molecules in the rafts platform leading to magnified signaling cascade. Contrastingly, perturbation the lipid rafts/caveolae dispersed Rap1 to the non-caveolar fractions, and therefore disrupting PACAP signaling.

4.2.8.3. PACAP-elicited transient Ras activation and the effect of modulation of plasma membrane glycosphingolipid level on its activation

Although EPAC activation enhanced PACAP-stimulated ERK1/2 phosphorylation (Fig 4.2.7.C) and subsequently promoted neurite extension of PC12 cells induced by PACAP (Fig 4.2.7.B), activation of EPAC itself did not induce neuritogenesis (Fig 4.2.7.A). Activating the EPAC-Rap1 signaling cascade was therefore not sufficient for neurite extension, and there must exist other pathway(s) which was (were) necessary for integrating the signals to generate the biological outcome. It has been reported that NGF required both Rap1- and Ras-dependent B-Raf activation to induce neurite extension (Lazarovici et al. 1998). Whether this happens in PACAP signaling is worth checking.

To address the above, Ras activation upon PACAP stimulation was further investigated in PC12 cells. Ras was rapidly activated upon PACAP addition (Fig 4.2.8.3.A), with a maximum level of activation occurring in the early phase of stimulation. However, PACAP-elicited Ras GTP-loading was transient and much weaker than that observed with NGF (Fig 4.2.8.3.A), which was consistent with the more dramatic neurite extension in NGF- than in PACAP-stimulated PC12 cells. These data indicate that Ras might have been involved in PACAP signaling.

Ras farnesylation is an absolute requirement for Ras membrane anchorage and transforming activity (Hancock et al. 1989; Casey et al. 1989; Gibbs 1991; Kato et al. 1995). Consistent with this notion, the potent Ras farnesyltransferase inhibitor FPTII significantly inhibited both PACAP-induced neurite extension (Fig 4.2.8.3.B) and ERK1/2 phosphorylation (Fig 4.2.8.3.C). Similar data from others (Busca et al. 2000) show that activation of ERK by cAMP was blocked by a dominant-negative Ras in melanocytes. These results further confirm that Ras had played a role in mediating PACAP signaling in PC12 cells, although it might not be directly activated by cAMP (Busca et al. 2000). In addition, modulating the rafts glycosphingolipid level by either GM1 or NB-DNJ affected the Ras GTP-loading elicited by PACAP (Fig 4.2.8.3.D). Ras activation was enhanced when plasma membrane was imbued with GM1 and was prohibited when membrane glycophingolipid level was decreased by NB-DNJ (Fig 4.2.8.3.D). Similar enhancement on Ras activity by GM1 might still exist in the

A.

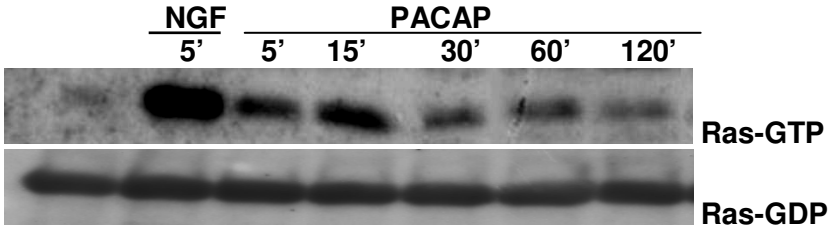

Fig 4.2.8.3. A. PACAP-stimulated Ras GTP loading. PC12 were incubated with or without (negative control) PACAP or NGF (positive control) for indicated time periods. The cells were harvested and performed Ras activation assay using EZ-detect Ras activation assay kit from Pierece. GTP-loaded Ras was affinity-precipitated using Ras binding domain GST-Raf1 and immunodetected with a Ras antibody (upper panel). The GDP-loaded Ras protein was shown in the lower panel.

B.

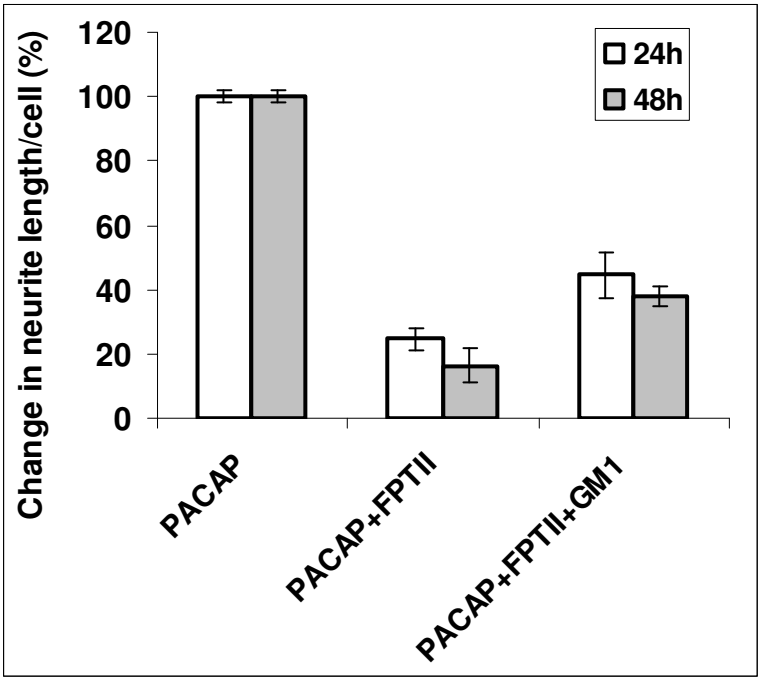

Fig 4.2.8.3. B. The inhibitory effect of FPTII on PACAP-induced neurite outgrowth and the influence of membrane GM1 in this process. PC12 cells incubated with PACAP in the absence or presence of FPTII (0.1 mM) or both FPTII and GM1. Phase contrast micrographs were taken 24 h and 48 h after PACAP was added. Neurite length/cell was quantified. The relative change was expressed as a percentage of the mean neurite length/cell for the cells treated with PACAP alone (100%), as described in Materials and Methods. Values are mean ± SD of at least three independent experiments.

C.

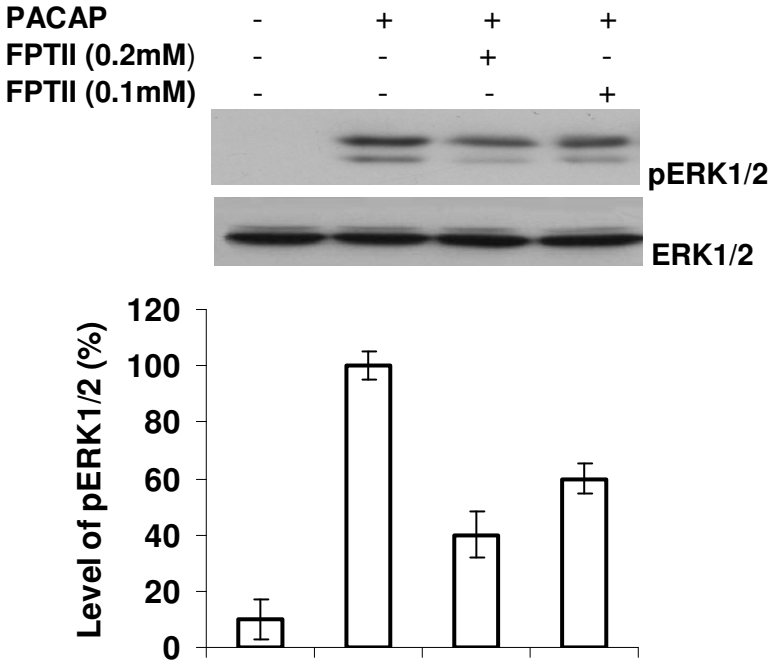

Fig 4.2.8.3. C. The inhibitory effect of FPTII on the ERK1/2 phosphorylation induced by PACAP. PC12 cells were treated with or without FPTII (0.1 mM or 0.2 mM) for 1 h before replacing the medium by that containing 100 nM PACAP. The samples were subjected to Western blotting using phosphospecific ERK1/2 (pERK1/2) antibody. Equal loading was verified by probing the blots with an anti-ERK1/2 (ERK1/2) antibody. In quantitative analysis, the phosphorylated ERK1/2 level in PACAP-treated PC12 cells was set as 100%. Values are mean ± SD of at least three independent experiments.

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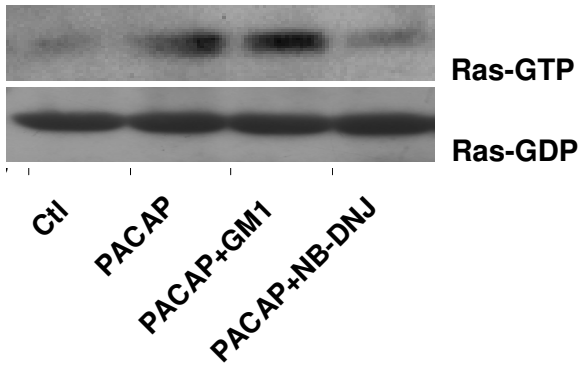


Fig 4.2.8.3. D. The effect of membrane glycosphingolipid level on the PACAP-induced Ras activation. PC12 cells were treated with or without GM1 or NB-DNJ followed by incubation with PACAP for 5 min. The cells were harvested and performed Ras activation assay as described.

presence of FPTII because the neurite outgrowth blocked by the latter was slightly relieved in the presence of GM1 (Fig 4.2.8.3.B). These data demonstrate that Ras activation, which relied on the integrity of rafts/caveolae, was involved in PACAP signaling in PC12 cells.

4.2.9. Phospholipase C, protein kinase C and intracellular Ca^{2+} elevation are involved in the PACAP-stimulated ERK1/2 activation and neuritogenesis of PC12 cells

In many cases, GPCRs are positively coupled to AC or phospholipase C (PLC). As shown previously, the interaction between PAC1R and AC upon PACAP treatment was facilitated (Fig 4.2.2.C). Whether PAC1R also stimulates PLC activity was investigated as below.

4.2.9.1. The effect of inhibition to PLC on PACAP-induced neuritogenesis and ERK1/2 activation as well as the influence of membrane glycosphingolipid

First, we check the effect of the specific PLC inhibitor U73122 on the differentiation of PC12 cells in the presence of PACAP. Fig 4.2.9.A shows that PACAP-elicited neurite outgrowth was inhibited by approximately 60-70% in the presence of 1 or 10 μM U73122 compared to untreated cells. It is of interest to note that the inhibitory

A.

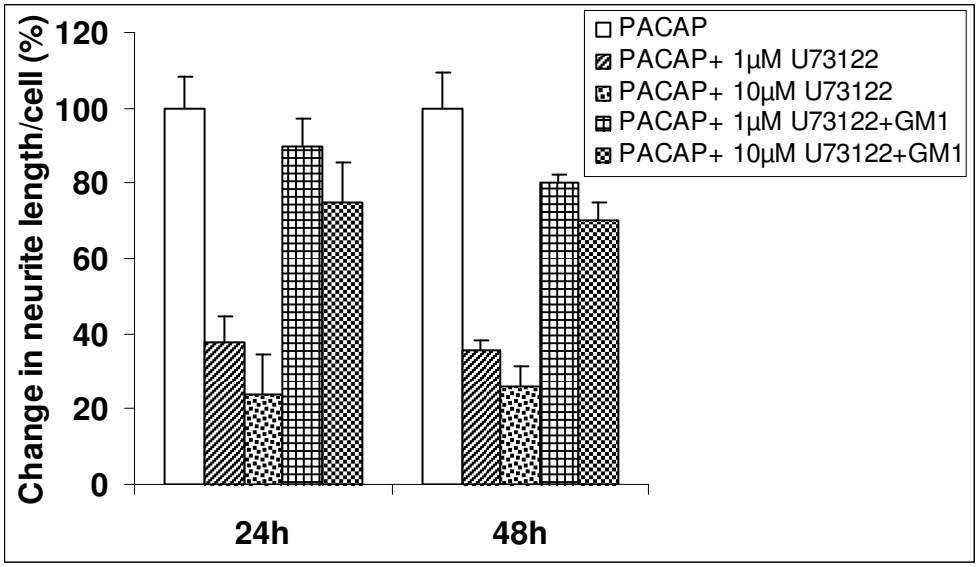


Fig 4.2.9. A. The inhibitory effect of PLC inhibitor U73122 on the PACAP-induced neurite outgrowth and the influence of exogenous GM1. PC12 cells were pretreated with or without different concentrations (1, 10 μM) of U73122 in the presence or absence of GM1 in PACAP containing medium. The phase contrast micrographs were taken at 24 h and 48 h after PACAP was added. The neurite length/cell was quantified as described. The relative change was expressed as a percentage of the mean neurite length/cell for the cells treated with PACAP alone (100%), as described in Materials and Methods. Values are mean ± SD of at least three independent experiments.

B.

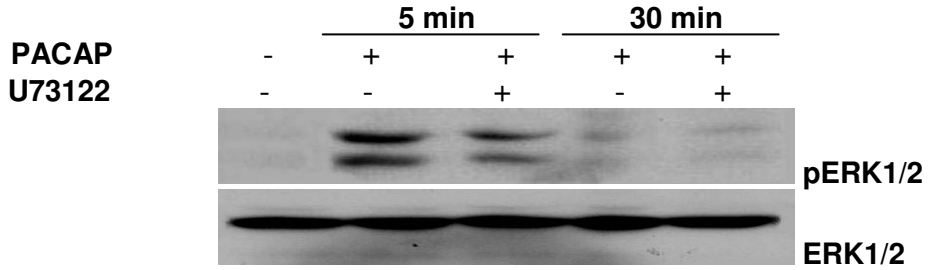


Fig 4.2.9. B. The inhibitory effect of U73122 on the PACAP-induced ERK1/2 activation. PC12 cells were treated with or without U73122 (1 μM) followed by incubation with PACAP for 5 min or 30 min respectively. The samples were subjected to Western blotting using a phosphospecific ERK1/2 (pERK1/2) antibody. Equal loading was verified by probing the blots with an anti-ERK1/2 (ERK1/2) antibody. The data are representative of three independent experiments.

effect of U73122 could be compensated, at least partly, by increase the membrane GM1 concentration. In addition, inhibition of PLC reduced the PACAP-stimulated ERK1/2 activation significantly at both time points of observation (Fig 4.2.9.B). These results suggest that besides elevation of cAMP, PLC activation was also necessary for the PACAP-elicited activation of ERK1/2 and neuritogenesis.

4.2.9.2. The role of PKC in PACAP-induced neuritogenesis and ERK1/2 activation as well as the influence of membrane glycosphingolipid

The involvement of protein kinase C (PKC) in PACAP signaling was investigated using the phorbol ester (PMA), a PKC activity modulator. Short-term PMA incubation has been reported to increase PKC levels, while long-term incubation was reported to downregulate PKC (Matthies et al. 1987; Singh et al 1994). In this study, short-term (1 h) incubation of PC12 cells with PMA alone did not result in significant growth of neurites (Fig 4.2.9.C and D), although such treatment is known to increase expression of developmentally regulated proteins (Balbi and Allen, 1994; Costello et al. 1990; Burry and Perrone-Bizzozero 1993; Perrone-Bizzozero et al. 1993; Reinhold and Neet 1989). However, PMA could enhance PACAP- (Fig 4.2.9.C) or db-cAMP- (a cAMP analog) (Fig 4.2.9.D) induced neurite outgrowth in PC12 cells. This enhancing effect of PMA was sustainable during the time period of observation (Fig 4.2.9.C), particularly for PACAP-stimulated cells. Taken together, these results show that activation of PKC could enhance the response of PC12 cells to PACAP or cAMP.

C.

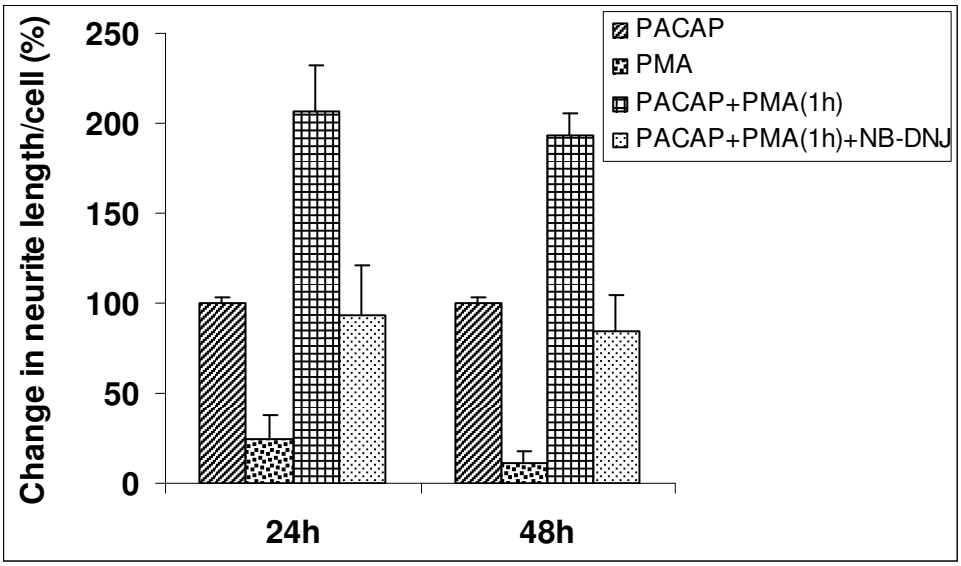


Fig 4.2.9. C. The effect of PKC activator, PMA, on PACAP-induced neurite outgrowth and the influence of membrane glycosphingolipids. PC12 cells were treated with PMA (0.1 μM) alone or pretreated with PMA for 1 h in the presence or absence of NB-DNJ before incubated with PACAP. The phase contrast micrographs were taken 24 h and 48 h after PACAP was added. The neurite quantification analysis was performed as described in Materials and Methods. Values are mean ± SD of at least three independent experiments.

D.

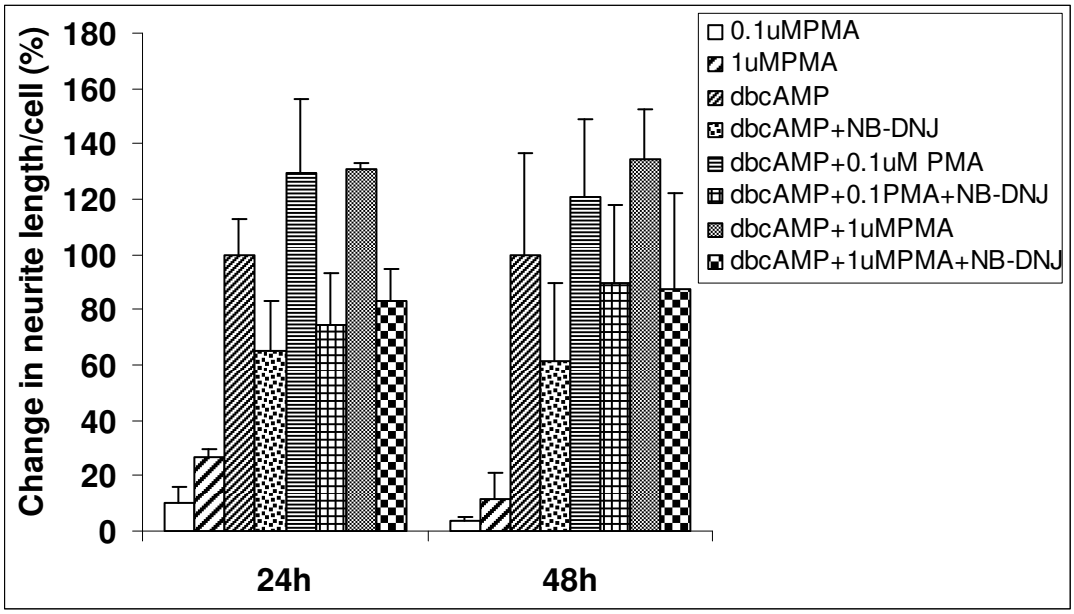


Fig 4.2.9. D. The effect of PKC activator, PMA, on the cAMP analog dbcAMP-induced neurite outgrowth and the influence of membrane glycosphingolipids. PC12 cells were pretreated with or without NB-DNJ for 24 h as described in Materials and Methods. Differently treated cells were then incubated with various concentrations (0.1 or 1 μM) of PMA for 1 h before the induction of 1 mM dbcAMP. The phase contrast micrographs were taken at 24 h, 48 h and 72 h time points after dbcAMP was added and neurite quantification analysis was performed. The relative change was expressed as a percentage of the mean neurite length/cell for the cells treated with PACAP alone (100%), as described in Materials and Methods. Values are mean ± SD of at least three independent experiments.

PKC might therefore incorporate with cAMP in PACAP signaling in PC12 cells.

To confirm the above, the effects of a specific PKC inhibitor, chelerythrine, which competes for the conserved catalytic sites of PKC (Herbert et al. 1990), and long-term incubation with PMA, which is known to downregulate PKC, on PACAP-induced neurite extension were investigated. As expected, pretreatment with chelerythrine inhibited PACAP-induced neurite outgrowth by 40% compared to the control (Fig 4.2.9.E). The inhibitory effect of prolonged PMA-incubation on PACAP-induced neuritogenesis happened only after 48 h, nevertheless, significant (Fig 4.2.9.E). Consistent with this, pretreatment with chelerythrine or long-term incubation with PMA significantly reduced ERK1/2 activation elicited by PACAP while the total ERK1/2 level was unaffected (Fig 4.2.9.I). On the other hand, short-term incubation with PMA increased the ERK1/2 activation significantly. On top of these, PKC activity was increased upon PACAP activation (Fig 4.2.9.H), indicative of the direct involvement of PKC in PACAP signaling. Taken together, these results suggest that activation of PKC was necessary for PACAP to efficiently stimulate ERK1/2 activation and subsequent neurite extension.

Finally, the effects of both PKC activator PMA and inhibitor chelerythrine on PACAP-induced neurite outgrowth of PC12 cells could be modulated by glycosphingolipid level (Figs 4.2.9.C, D and F), consistent with the importance of lipid rafts/caveolae in PACAP signaling.

4.2.9.3. The role of Ca^{2+} in PACAP-induced neuritogenesis and ERK1/2 activation as well as the influence of membrane glycosphingolipid

Since inositol 1, 4, 5-trisphosphate (IP_3) formed by the action of PLC can activate Ca^{2+} release from intracellular Ca^{2+} stores, the possible involvement of intracellular Ca^{2+} was then investigated. Perturbation of intracellular Ca^{2+} levels by either prolonged or short-term exposure to thapsigargin (THG), which depletes intracellular Ca^{2+} stores, dramatically retarded the neurite outgrowth elicited by PACAP (Fig 4.2.9.G). Apparently, prolonged exposure (24 h) to THG inhibited neurite extension more effectively than short-term exposure (1 h), a reflection of the time period needed for complete depletion of the intracellular Ca^{2+} store. Moreover, PACAP is known to stimulate transient Ca^{2+} influx (Osipenko et al. 2000). To analyze the involvement of Ca^{2+} influx in PACAP-stimulated neurite extension, extracellular Ca^{2+} was removed by the Ca^{2+} chelator ethylene glycol bis (2-aminoethyl-ether)-N, N, N', N'-tetraacetic acid (EGTA). Pretreatment of cells with EGTA significantly attenuated the neurite extension induced by PACAP (Fig 4.2.9.F), suggesting the involvement of Ca^{2+} influx in PACAP-induced neurite extension. Moreover, both THG and EGTA inhibited the increase in ERK1/2 phosphorylation induced by PACAP, while total ERK1/2 level was unaffected (Fig 4.2.9.I). Taken together, these data suggest that PACAP might cause a change in intracellular Ca^{2+} concentration, resulting from mobilization of Ca^{2+}

from intracellular store and also Ca^{2+} influx, and that this rise in intracellular Ca^{2+} was likely part of PACAP signaling.

In addition, retardation of PACAP-induced neurite outgrowth by THG and EGTA could be partially restored by GM1 (Fig 4.2.9.F and G), suggesting that the lipid rafts/caveolae-mediated PACAP signaling, which leads to neurite extension in PC12 cells might be modulated, at least partly, by crosstalk with intracellular Ca^{2+}.

4.2.10. Glycogen synthase kinase 3β (GSK3β) is involved in lipid rafts/caveolae-mediated PACAP signaling in PC12 cells

Glycogen synthase kinase 3β (GSK3β) is a highly conserved Ser/Thr kinase originally identified as a negative regulator of glycogen synthesis, but is more recently implicated in signaling pathways important for early CNS patterning, neuronal survival (Mingtao et al. 2000) as well as differentiation (Williams and Harwood 2000). It is one of the two isoforms of mammalian GSK3 which are encoded by distinct genes, namely GSK3α (51 kDa) and GSK3β (47 kDa) (Woodgett 1990). GSK3 (α and β) is highly expressed in developing brain, where its expression correlates with the period of active neurite remodeling (Woodgett 1990; Takahashi et al. 1994; Leroy and Brion 1999). Accordingly, the role of GSK3 in regulating neurite morphology has been intensively studied (Eickholt et al. 2002; Sayas et al. 2002; Zhou et al. 2004; Yoshimura et al. 2005; Jiang et al. 2005). GSK3β has two splice

E.

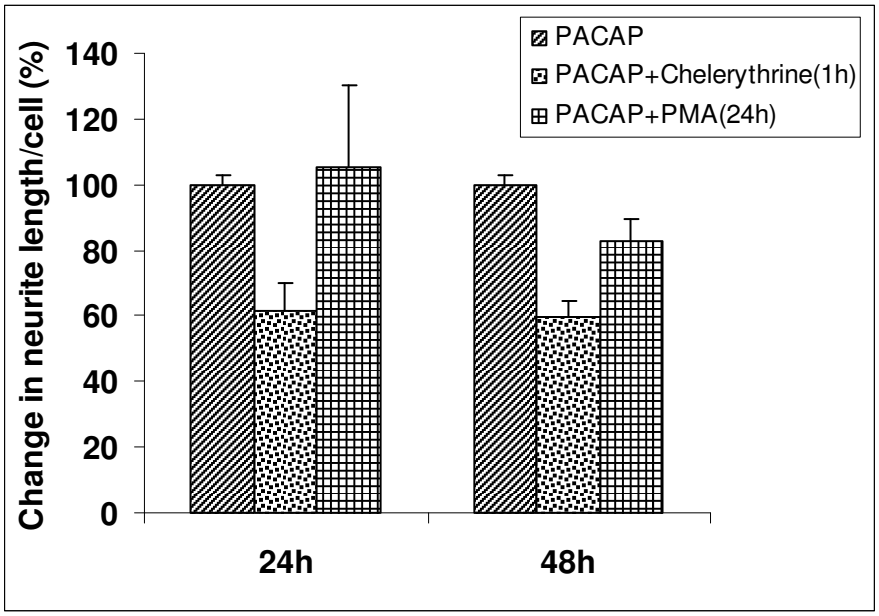


Fig 4.2.9. E. The effect of PKC activity on the PACAP-induced neurite outgrowth. PC12 cells were pretreated with chelerythrine (10 μM, 1 h), or PMA (0.1 μM, 24 h) before incubated with PACAP. The phase contrast micrographs were taken at 24 h and 48 h after PACAP was added and neurite quantification analysis was performed. The relative change was expressed as a percentage of the mean neurite length/cell for the cells treated with PACAP alone (100%), as described in Materials and Methods. Values are mean ± SD of at least three independent experiments.

F.

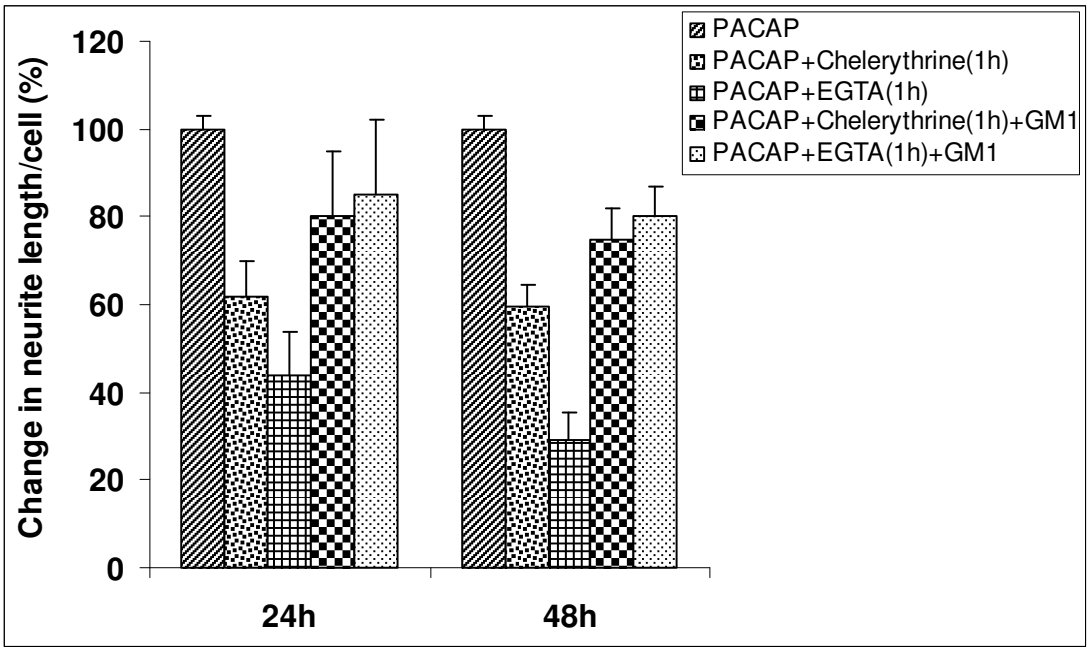


Fig 4.2.9. F. The effect of glycosphingolipid on the PKC and Ca^{2+} treated PC12 cells induced by PACAP. PC12 cells were pretreated with or without GM1 (100 µM, 24 h) followed by treated with chelerythrine (10 µM, 1 h) or EGTA (5 mM, 1 h). The phase contrast micrographs were taken at 24 h and 48 h after PACAP was added and neurite quantification analysis was performed. The relative change was expressed as a percentage of the mean neurite length/cell for the cells treated with PACAP alone (100%), as described in Materials and Methods. Values are mean ± SD of at least three independent experiments.

G.

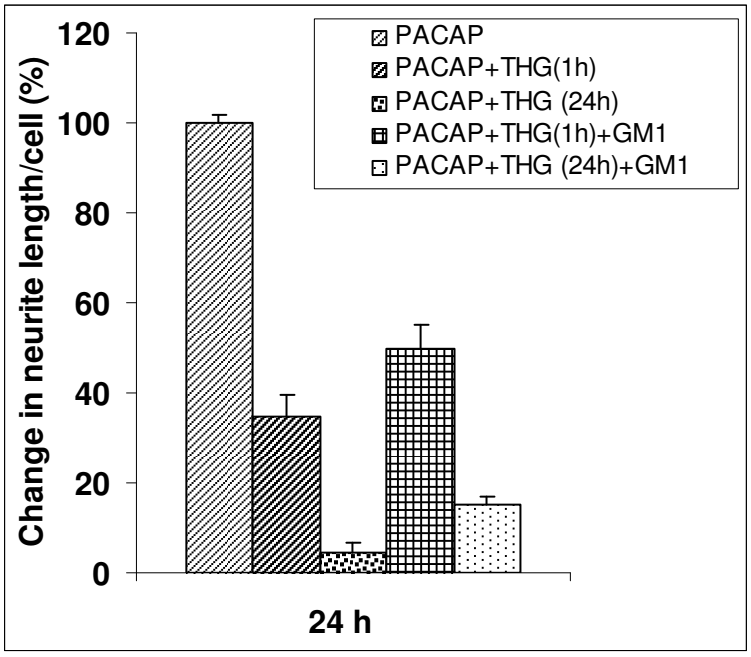


Fig 4.2.9. G. The effect of intracellular Ca^{2+} on the PACAP-induced neurite outgrowth. PC12 cells were pretreated with thapsigargin (THG) (5 μM) for either 1 h or 24 h in the presence or absence of exogenous GM1 before incubated with PACAP. The phase contrast micrographs were taken 24 h after PACAP was added and neurite quantification analysis was performed accordingly. Values are mean ± SD of at least three independent experiments.

H.

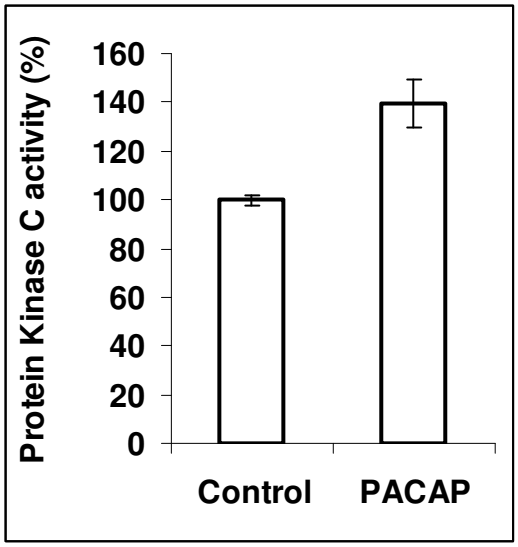


Fig 4.2.9. H. PACAP-stimulated PKC activation. PC12 cells were stimulated with or without (control) PACAP for 5 min before harvesting for PKC activity assay as described in Material and Methods. Values are mean ± SD of at least three independent experiments.

I.

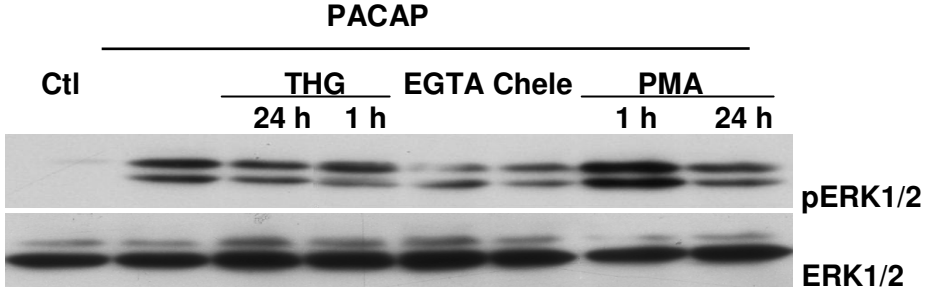


Fig 4.2.9. J. The effect of PKC and Ca^{2+} on the PACAP-induced ERK1/2 phosphorylation. PC12 cells were treated with thapsigargin (THG) (5 µM) or PMA (0.1 µM) for 1 h or 24 h, or incubated with EGTA (5 mM), PKC inhibitor Chelerythrine (Chele) (10 µM) for 1 h before brief incubation with PACAP for 5 min. The cells were harvested and subjected to Western blotting using phosphospecific ERK1/2 (pERK1/2) antibody. Equal loading was verified by probing the blots with anti- ERK1/2 (ERK1/2) antibody.

variants (Mukai et al. 2002; Schaffer et al. 2003). It has constitutive inhibitory activity, that inactivation or activation of GSK-3β promotes or retards neurite outgrowth, respectively. GSK3β activity is under both positive and negative regulation by upstream kinases. Phosphorylation of Tyr-216 within its kinase domain promotes activity, whereas Ser-9 phosphorylation is inhibitory (Wang et al. 1994). Various kinases have been implicated in mediating serine phosphorylation and inactivation of GSK3β, including phosphatidylinositol 3-kinase (PI3K)-regulated Akt/PKB, protein kinase A and protein kinase C (Cross et al. 1995; Fang et al. 2000; Fang et al. 2002). Lithium inhibits the activity of GSK3β by increasing its phosphorylation on Ser-9 (Kirshenboim et al. 2004), and sodium valproate also modestly increases the phosphorylation of GSK3β on Ser-9 (De Sarno et al. 2002). On the other hand, inhibitors of PI3K, LY294002 and wortmannin, are indirect activators of GSK3β as they abolished lithium-induced Ser-9 phosphorylation of GSK3β in both HEK 293 and PC12 cells (Kirshenboim et al. 2004). The ability of GSK3β to regulate neuronal architecture is thought to rely on its ability to phosphorylate microtubule-binding proteins, particularly the neuron-specific proteins tau (Hanger et al. 1992; Wagner et al. 1996), MAP1B (Trivedi et al. 2005; Goold and Gordon-Weeks 2004), CRMP-2 (Yoshimura et al. 2005), and the widely expressed adenomatous polyposis coli protein (APC) (Goold and Gordon-Weeks 2004). Phosphorylation of these proteins by GSK3β promotes microtubule disassembly and destabilization (Zhou and Snider 2005), and provided a strong rationale for developing selective GSK3β inhibitors for the treatment of neuropathologies (Cohen

and Goedert 2004). Indeed, phosphorylation of MAP1B, which maintains the population of unstable microtubules in growing axons and dephosphorylation of APC, which increases stable microtubules, have been reported during NGF-stimulated PC12 cell differentiation (Goold and Gordon-Weeks 2001; 2004; Zhou et al. 2004). However, the functions of GSK3β in differentiation induced by PACAP and the underlying mechanisms of regulation are still lacking. In particular, whether phosphorylation of GSK3β Ser-9 occurs during PACAP-induced neuritogenesis in PC12 cells is not known so far.

4.2.10.1. The role of GSK3β in PACAP-induced neurite outgrowth and the influence of plasma membrane glycosphingolipid

Not surprisingly, lithium, an inhibitor of GSK3β activity, remarkably promoted PACAP-evoked neurite outgrowth at the concentration of 10 mM (Fig 4.2.10.A). This stimulatory effect was attenuated by inhibition of either the plasma membrane glycosphingolipid level by NB-DNJ or cholesterol level by U18666a (Fig 4.2.10.A). Similarly, another GSK3β activity inhibitor, valproate, enhanced the PACAP-induced neurite extension of PC12 cells (Fig 4.2.10.B). Again, perturbation of the plasma membrane rafts/caveolae by NB-DNJ or U18666a decreased the accelerative effect of valproate on neurite outgrowth (Fig 4.2.10.B). These data indicate that inhibition of GSK3β activity did increase the neurite extension elicited by PACAP. To further confirm this, the function of GSK3β was modulated by PI3K inhibitors, wortmannin

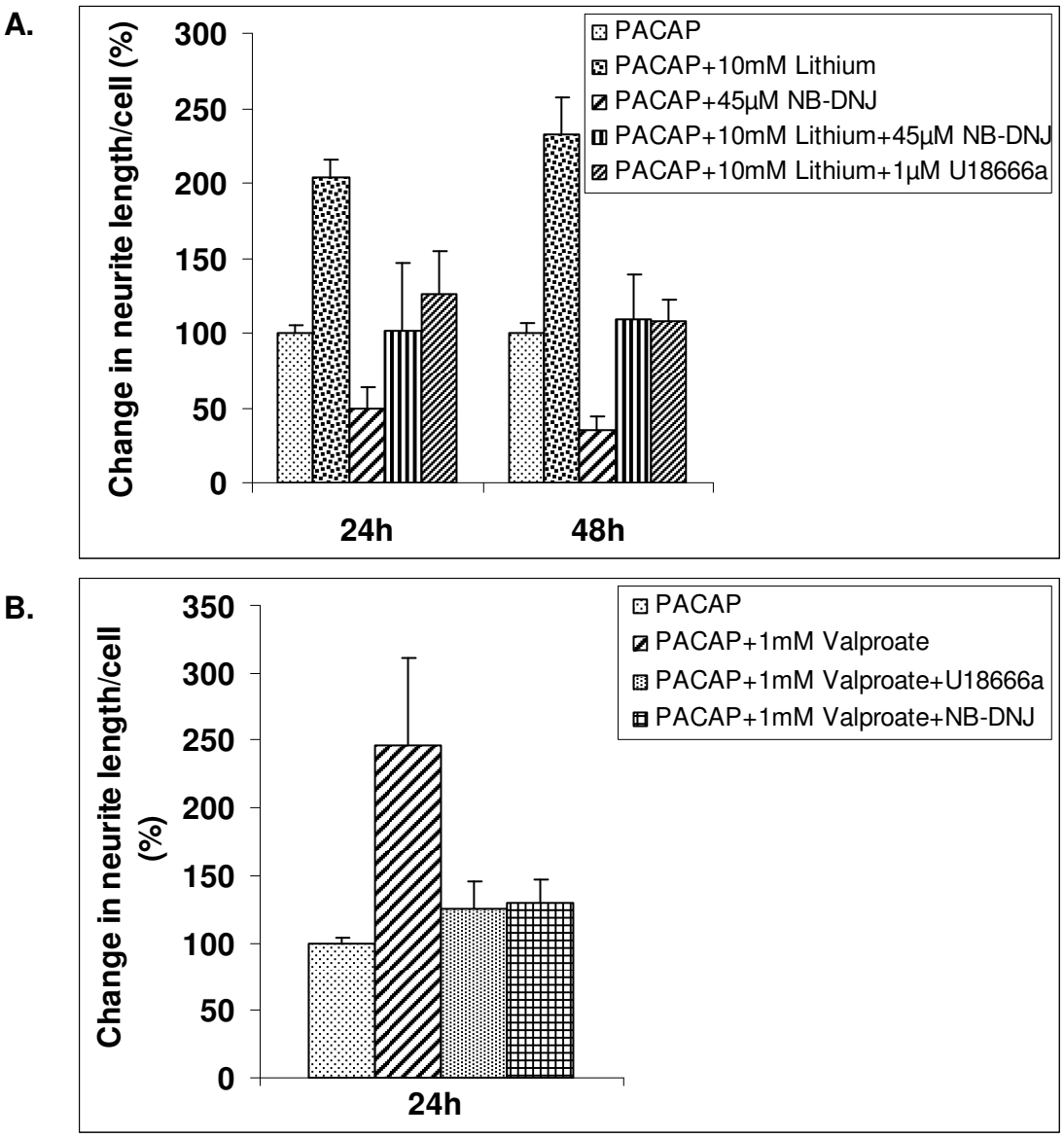


Fig 4.2.10. The potentiation effect of GSK3β inhibitors on the PACAP-induced neurite outgrowth and the influence of glycosphingolipid in this process. PC12 cells were treated with or without NB-DNJ or U18666a for 24 h before incubated with or without 10 mM lithium **(A)** or 1 mM valproate **(B)** in the complete medium for 1 h. Phase contrast micrographs were taken at the indicated time points after PACAP was added and neurite quantification was performed. The relative change was expressed as a percentage of the mean neurite length/cell for the cells treated with PACAP alone (100%), as described in Materials and Methods. Values are mean ± SD of at least three independent experiments.

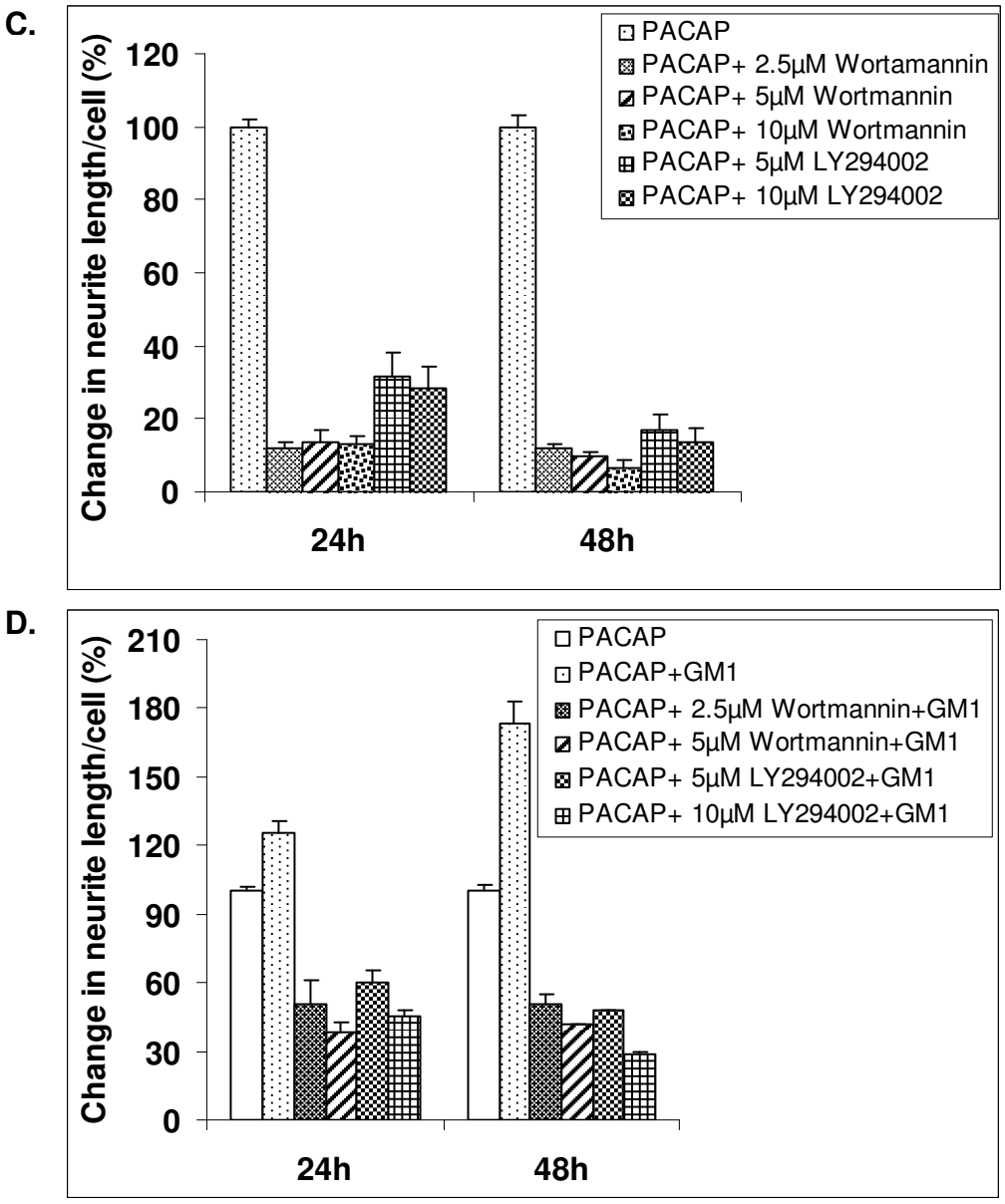


Fig 4.2.10. The inhibitory effect of specific PI3K inhibitors, indirect activators of GSK3β, on the PACAP-induced neurite outgrowth and the influence of glycosphingolipid in this process. PC12 cells were incubated with **(D)** or without **(C)** GM1 for 24 h before treated with or without various concentrations of wortmannin or LY294002 as indicated, in the complete medium for 1 h. Phase contrast micrographs were taken at 24 h and 48 h time points after PACAP was added and neurite quantification was performed. The relative change was expressed as a percentage of the mean neurite length/cell for the cells treated with PACAP alone (100%), as described in Materials and Methods. Values are mean ± SD of at least three independent experiments.

and LY294002, which are indirect activators of GSK3β. Both wortmannin (2.5, 5, 10 μM) and LY294002 (5, 10 μM) blocked PACAP-induced neurite outgrowth of PC12 cells (Fig 4.2.10.C). The enhancing effect of GM1 on neurite extension excited by PACAP was also attenuated in the presence of either wortmannin or LY294002 (Fig 4.2.10.D). Activation of GSK3β therefore reduced neurite outgrowth stimulated by PACAP. Taken together, these data clearly demonstrate that GSK3β was most likely involved in PACAP-elicited neuritogenesis of PC12 cells, which was mediated by plasma membrane lipid rafts/caveolae.

4.2.10.2. The effect of perturbation of lipid rafts/caveolae on PACAP-induced GSK3β phosphorylation

PACAP-induced phosphorylation of GSK3β was next analyzed using an antibody specifically recognizing phospho-Ser-9 GSK3β (Fig 4.2.10.E). Clearly, PACAP significantly increased the level of GSKB3β phosphorylation on Ser-9, supporting the notion that GSKB3β plays a role in PACAP signaling. In addition, both GM1 and cholesterol-methyl-β-cyclodextrin, which promoted PACAP-induced neurite outgrowth, enhanced the phosphorylation of GSK3β on Ser-9 elicited by PACAP. On the other hand, NB-DNJ or U18666a, which attenuated PACAP-induced neurite extension, decreased the GSK3β phosphorylation. The total amount of GSK3β was not affected (Fig 4.2.10.E). These results confirm that GSK3β was phosphorylated upon PACAP stimulation and the level of inhibitory phosphorylation of GSK3β was

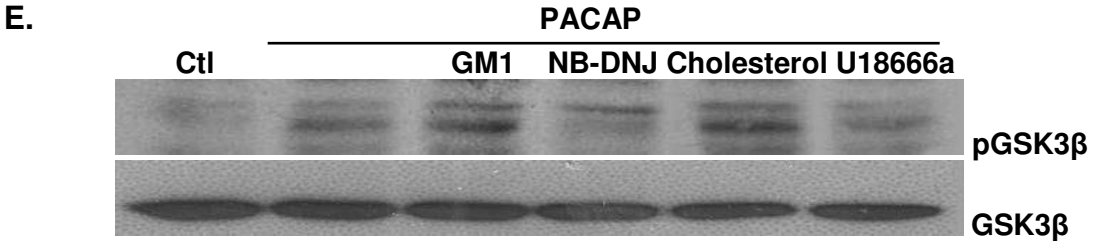


Fig 4.2.10. E. The effect of perturbation of the components of lipid rafts/caveolae on the PACAP-induced GSK3β phosphorylation. PC12 cells were treated with GM1, NB-DNJ, Cholesterol-mβCD (Cholesterol) or U18666a for 24 h followed by incubated with PACAP for 5 min. Western blotting was performed using a phosphospecific GSK3β (pGSK3β) antibody. Equal loading was verified by probing the blots with an anti-GSK3β antibody.

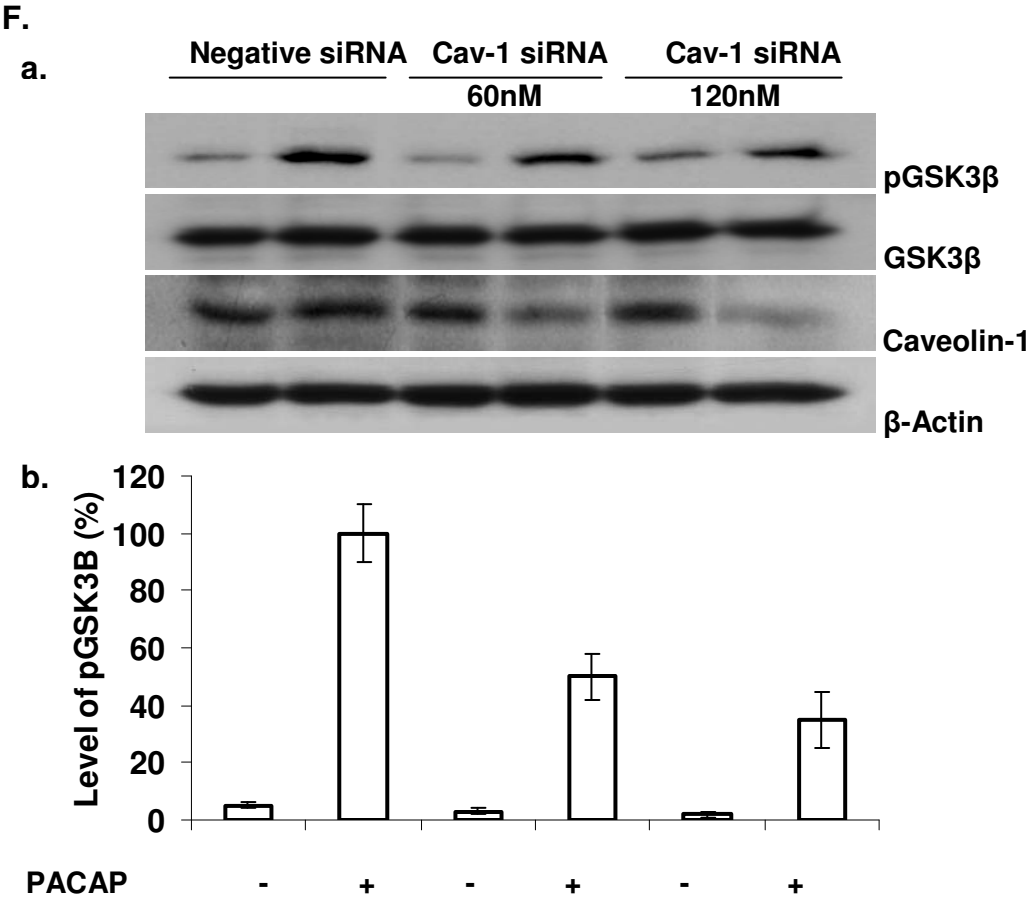


Fig 4.2.10. F. The effect of caveolin-1 siRNA on the PACAP-induced GSK3β phosphorylation. (a). PC12 cells were transfected with control or caveolin-1 siRNA, followed by incubated with or without PACAP for 5 min. The samples were subjected to Western blotting with a phosphospecific GSK3β (pGSK3β) antibody. Equal loading was verified by probing the blot with an anti-GSK3β antibody. **(b).** Quantitation the relative change of pGSK3β obtained in (a).

correlative with the extent of neurite outgrowth. In addition, this phosphorylation appeared to be regulated in the process of PACAP signaling through lipid rafts/caveolae. Consistent with this notion, perturbation of lipid rafts/caveolae by caveolin-1 siRNA inhibited PACAP-stimulated phosphorylation of GSK3β, while total GSK3β level was not changed (Fig 4.2.10.F). These results verify that phosphorylation of GSK3β by PACAP signaling was modulated by the integrity and proper function of lipid rafts/caveolae.

4.2.10.3. The effect of PKC and Ca^{2+} on PACAP-induced GSK3β phosphorylation

To further determine the upstream events that regulate the activity of GSK3β, PKC and Ca^{2} elevation were inhibited by pharmacological blockers and the GSK3β phosphorylation level was examined by Western blot. Inhibition of PKC by chelerythrine and prolonged incubation with PMA decreased GSK3β phosphorylation level, while total GSK3β was not affected (Fig 4.2.10.G). These results imply that the activity of GSK3β was regulated by PKC. Similarly, depletion of Ca^{2+} store by THG or sequestration of the extracellular Ca^{2+} by EGTA reduced the GSK3β phosphorylation level (Fig 4.2.10.G). The inhibitory effect of THG with long-term incubation (24 h) was more intensive than with short-term incubation (1 h) (Fig 4.2.10.G), suggesting that the concentration of intracellular Ca^{2+} has an impact on the level of phosphorylation of GSK3β. Taken together, these data indicate that PACAP-

elicited GSK3β phosphorylation on Ser-9 might be modulated by PKC activation and Ca^{2+} mobilization.

4.2.10.4. The effect of Rap1 on PACAP-induced GSK3β phosphorylation

It has been shown that PKC and Ca^{2+}, activated by PACAP, might synergize with cAMP elevation which in turn loaded Rap1 with GTP, thereby inducing neurite generation in PC12 cells. Thus, whether the activity of GSK3β is also modulated by cAMP and how it was regulated were further questions to be asked. To investigate these, Rap1 expression was silenced by siRNA. As illustrated in Fig 4.2.10.H, PC12 cells transfected with Rap1 siRNA showed a marked reduction (≈70%) in the level of Rap1 protein expression compared with that in control cells. As a result, the level of GSK3β phosphorylation, although not altered at the very early stage of PACAP induction (5 min), decreased significantly thereafter and the inhibitory effect of Rap1 siRNA on the phosphorylation of GSK3β was sustainable for at least 2 h (Fig 4.2.10.H). In addition, suppression of Rap1 also led to a marked and sustained reduction of ERK1/2 phosphorylation (Fig 4.2.8.1 B). These results suggest that Rap1 activation could be an upstream event of GSK3β phosphorylation in PACAP signaling. Moreover, changes to GSK3β and ERK1/2 phosphorylation upon Rap1 regulation were consistent, indicating the possible correlation between these effector proteins in the PACAP signaling transduction cascade leading to neurite extension.

G.

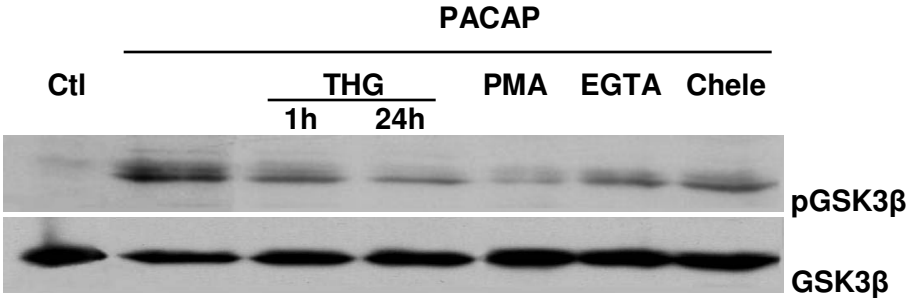


Fig 4.2.10. G. The effect of PKC and Ca^{2+} on the PACAP-induced GSK3β phosphorylation. PC12 cells were treated with thapsigargin (THG) (5 μM) for either 1 h or 24 h or incubated with PMA (0.1 μM), EGTA (5 mM) or Chelerythrine (Chele) (10 μM) for 1 h before brief incubation with PACAP for 5 min. The cells were harvested and subjected to Western blotting using a phosphospecific GSK3β (pGSK3β) antibody. Equal loading was verified by probing the blots an anti-GSK3β (GSK3β) antibody. The bands are representative of three independent experiments.

H.

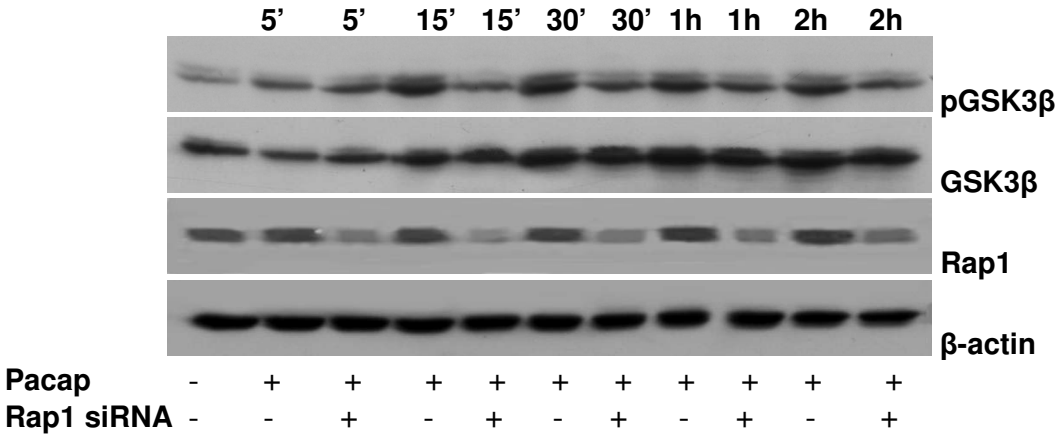


Fig 4.2.10. H. The effect of Rap1 siRNA on the phosphorylation of GSK3β. PC12 cells were transfected with control siRNA or Rap1 siRNA for 48 h before incubated with PACAP (100 nM) for the indicated time periods and then harvested. The whole cell lysates were subjected to Western blotting with phosphospecific GSK3β (pGSK3β) and anti-GSK3β (pGSK3β) antibodies, respectively. The efficiency of siRNA was evaluated 48 h after transfection using an anti-Rap1 antibody and equal loading was revealed by β-actin. The bands are representative of three independent experiments.

4.2.10.5. The effect of GSK3β on PACAP-induced ERK1/2 activation

Whether ERK1/2 and GSK3β phosphorylation were correlated in PACAP signaling, or whether ERK1/2 phosphorylation was the downstream (or upstream) event of GSK3β phosphorylation was examined. Different GSK3β inhibitors and activators were used to treat the PC12 cells. GSK3β and ERK1/2 phosphorylation levels were assured by Western blot. Inhibition of GSK3β constitutive activity by either lithium or valproate, which enhanced PACAP-induced GSK3β Ser-9 phosphorylation (Fig 4.2.10.I, middle lower panel) potentiated PACAP-elicited ERK1/2 phosphorylation at both Thr-202 and Tyr-204 (Fig 4.2.10.I, upper panel). Contrastingly, activating of GSK3β by LY294002 or wortmannin, which reduced GSK3β phosphorylation level to the basal level (Fig 4.2.10.I, middle lower panel) decreased ERK1/2 activation by PACAP (Fig 4.2.10.I, upper panel), while total ERK1/2 was not affected (Fig 4.2.10.I, middle lower panel). In the absence of PACAP, lithium, valproate, LY294002 and wortmannin alone did not elicit ERK1/2 activation above basal level (Fig 4.2.10.I, upper panel). These data provide evidence that PACAP-evoked ERK1/2 activation was regulated by GSK3β phosphorylation. Consistent with this, the MEK1/2 inhibitor PD98059 did not change PACAP-elicited GSK3β phosphorylation (Fig 4.2.10.J). Taken together, ERK1/2 activation was most likely the downstream event of GSK3β phosphorylation in PACAP-induced differentiation of PC12 cells.

I.

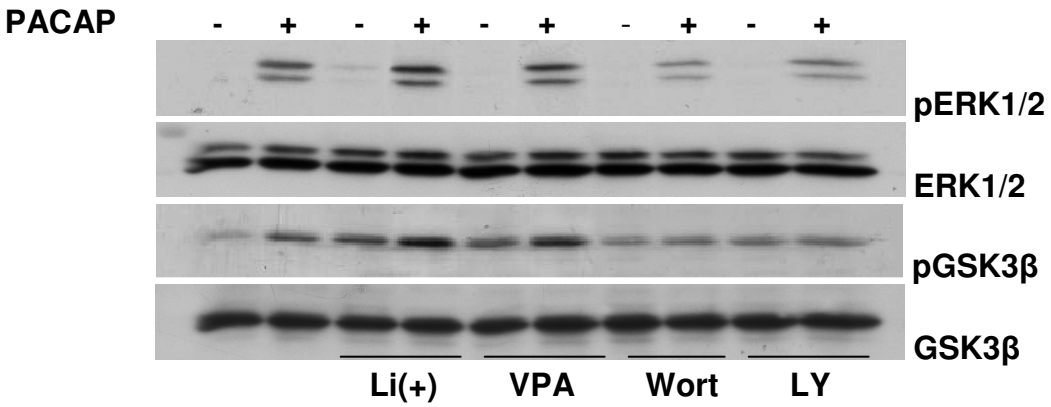


Fig 4.2.10. I. The effect of GSK3β on the phosphorylation of ERK1/2. PC12 cells were treated with either GSK3β activity activators (LY294002 and wortmannin) or inhibitors (lithium and valproate) for 1 h before incubated with PACAP for 5 min and harvested. The whole cell lystates were subjected to Western blotting with a phosphospecific ERK1/2 (pERK1/2) antibody or a phosphospecific GSK3β (pGSK3β) antibody. Equal loading was verified by probing the blots with an anti-ERK1/2 (ERK1/2) or an anti-GSK3β (GSK3β) antibody. The bands are representative of three independent experiments.

J.

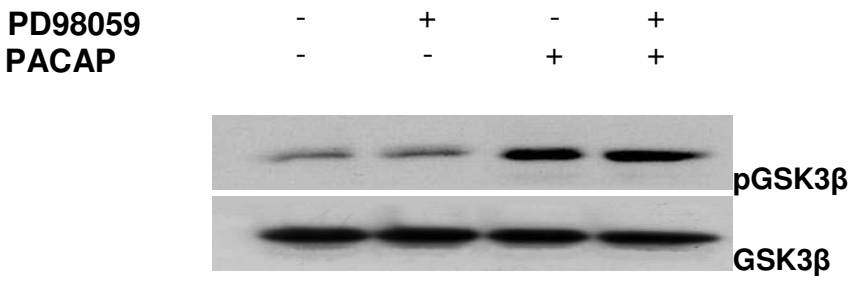


Fig 4.2.10. J. The effect of ERK1/2 activity on the phosphorylation of GSK3β. PC12 cells were treated with or without PD98059 (100 μM) for 1 h before incubated with or without PACAP (100 nM) for 5 min. The cells were harvested and subjected to Western blotting with a phosphospecific GSK3β (pGSK3β) antibody. Equal loading was verified by probing the blots with an anti-GSK3β (GSK3β) antibody. The bands are representative of three independent experiments.

4.2.10.6. The effect of Ras on PACAP-induced GSK3β phosphorylation

Lastly, the effect of Ras on the phosphorylation of GSK3β induced by PACAP was examined. Unlike the effect of Rap1, suppression of Ras activity by specific Ras farnesylation inhibitor FPTII at both concentrations applied (0.1 mM and 0.2 mM), did not affect either the phosphorylation or the total level of GSK3β elicited by PACAP (Fig 4.2.10.K). These results demonstrate that, unlike the positive effect of Rap1 on GSK3β phosphorylation, PACAP-induced GSK3β phosphorylation was not modulated by Ras activity in PC12 cells.

Taken together, the above data demonstrate that GSK3β was involved in PACAP signaling pathways in PC12 cell by modulating ERK1/2 activation. On the other hand, GSK3β phosphorylation was downstream event of Rap1 and PKC activation as well as Ca^{2+} mobilization, however, it was independent of Ras activation. On top of this, perturbation of lipid rafts/caveolae affected PACAP-induced GSK3β phosphorylation in PC12 cells, indicative of the functional association of phosphorylation of GSK3β by PACAP with the membrane microdomains lipid rafts/caveolae.

K.

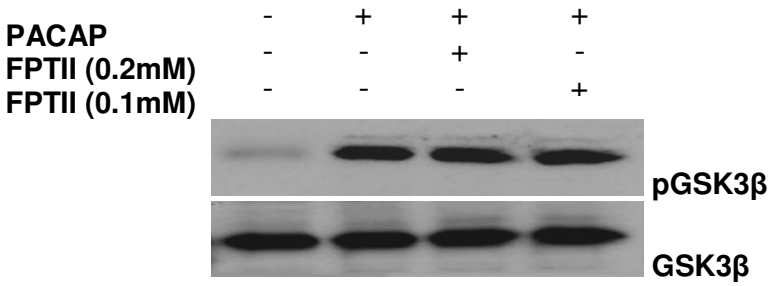


Fig 4.2.10. K. The effect of inhibition of Ras on the PACAP-induced GSK3β phosphorylation. PC12 cells were treated with different concentrations (0.1, 0.2 mM) of Ras process inhibitor FPTII for 1 h before incubated with PACAP for 5 min. The cells were harvested and subjected to Western blotting with a phosphospecific GSK3β (pGSK3β) antibody. Equal loading was verified by probing the blots with an anti-GSK3β (GSK3β) antibody. The bands are representative of three independent experiments.

4.2.11. Perturbation of the lipid rafts/caveolae by caveolin-1 siRNA, which inhibits the PACAP-elicited neurite extension, increases ERK1/2 activation

ERK1/2 are members of MAP Kinases which are Ser/ Thr kinases involved in regulating of a variety of cellular functions, such as cell proliferation, growth, differentiation and apoptosis (Davis 1993; Nishida and Gotoh 1993; Lewis et al. 1998; Chang and Karin 2001; Pearson et al. 2001). Sustained activation of ERK1/2 is required for full neurite outgrowth induced by several factors (Perron and Bixby 1999; Yaka et al. 1998; Perron and Bixby 1999). Indeed, ERK1/2 phosphorylation was found to be essential in the process of differentiation in PC12 cells stimulated by PACAP (Fig 4.2.5.2). However, the level of activated ERK1/2 stimulated by PACAP was attenuated by perturbation of the integrity of lipid rafts/caveolae when the major lipid components were removed (Fig 4.2.5.3), which in turn, led to inhibited neurite outgrowth (Fig 3.2.1.1). In the studies that follow, the effects of caveolin-1 siRNA, also known to disrupt the lipid rafts/caveolae, on the PACAP-elicited ERK1/2 activity are the issues to be further addressed.

4.2.11.1. The effect of perturbation of lipid rafts/caveolae by caveolin-1 siRNA on PACAP-induced ERK1/2 activation

As shown in Fig 4.2.11.A, the caveolin-1 expression level was remarkably inhibited (≈70%) in cells transfected with caveolin-1 siRNA compared to cells transfected with siRNA of random sequences or untreated cells. Interestingly, PACAP-elicited ERK1/2 activation was enhanced by a decrease in caveolin-1 expression, with more remarkable effect in the early stage of PACAP treatment (Fig 4.2.11.A and B), but the total ERK1/2 level was not affected. These data indicate that perturbation of lipid rafts integrity by caveolin-1 siRNA could increase the PACAP-evoked ERK1/2 activation and this effect was sustainable. These results appeared to be in contradiction to what found before. Although the reason for the increase of the ERK1/2 phosphorylation upon caveolin-1 siRNA transfection was still vague, the discrepancy might be explained by the fact that cavoelin-1 possesses transformation prohibitive activity by binding to and inactivating the signaling molecules including ERK1/2 (Okamoto et al. 1998). Therefore, when the caveolin-1 was downregulated, this inhibitory effect of caveolin-1 on the ERK1/2 was removed. Moreover, this effect on the increase in ERK1/2 phosphorylation might surpass the opposite effect of perturbation of membrane lipid rafts/caveolae caused by caveolin-1 siRNA on ERK1/2 phosphorylation.

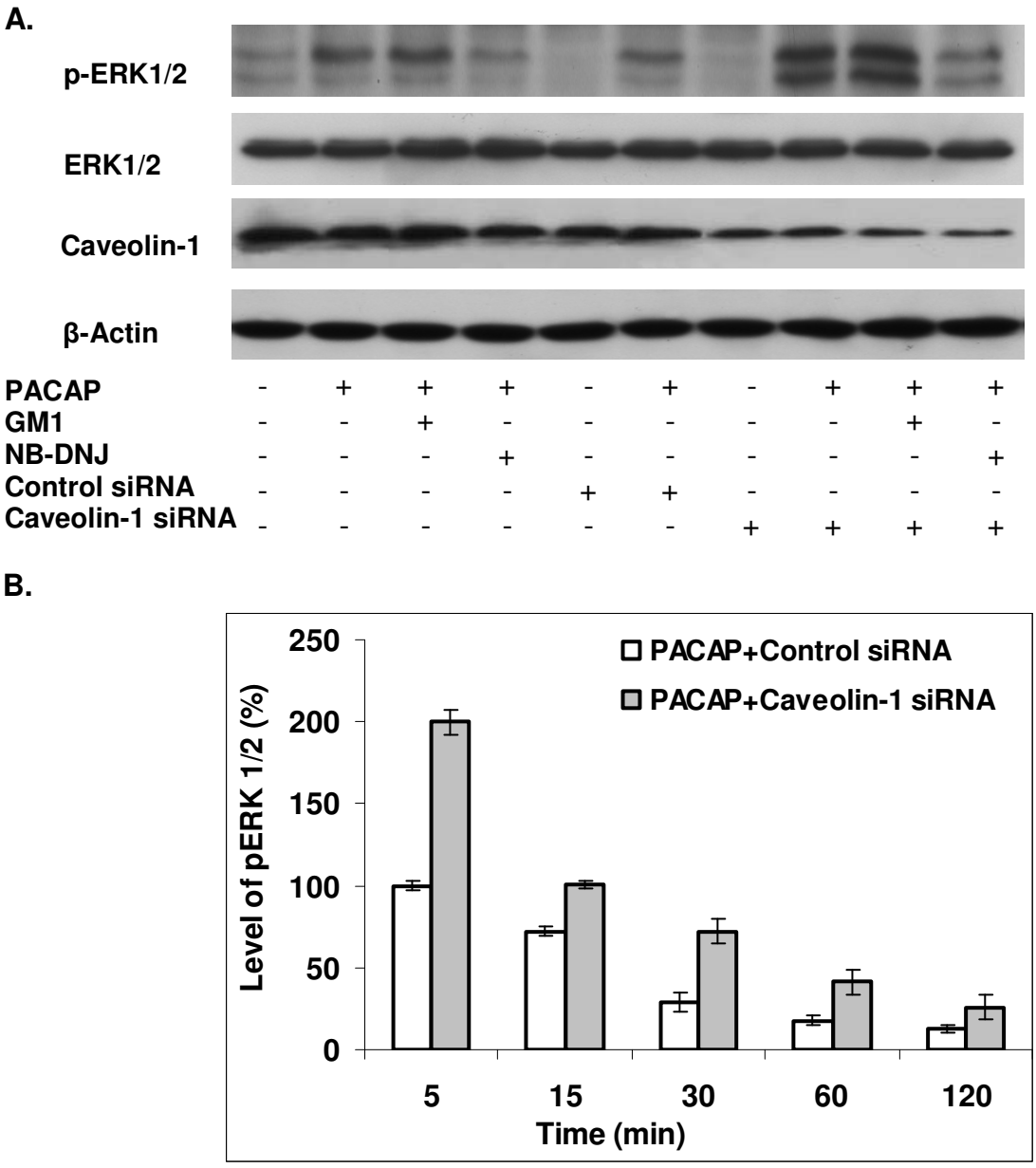


Fig 4.2.11. The effect of caveolin-1 siRNA on the PACAP-induced ERK1/2 activation and on the function of membrane glycosphingolipids. PC12 cells were treated with or without (control) caveolin-1 siRNA or control siRNA in the presence or absence of GM1 or NB-DNJ, followed by incubation with or without PACAP for 5 min. (**A**). The cells were harvested and subjected to Western blotting with pERK1/2 and ERK1/2 antibodies. Densitometry determination of pERK1/2 levels in control and caveolin-1 siRNA-treated cells in the presence of PACAP for the indicated time periods was shown in (**B**). The caveolin-1 expression level was evaluated 48 h after the caveolin-1 siRNA transfection (β-actin as loading control). The bands are representative of three independent experiments.

4.2.11.2. The effect of caveolin-1 siRNA on the ERK1/2 nuclear translocation elicited by PACAP

It is known that ERK1/2 must localize to the nucleus in order to execute its mitogenic function such as causing proliferation and differentiation (Brune et al. 1999). Many studies have reported that activation of the ERK1/2 pathway by diverse agonists results in its nuclear translocation of the active kinase (Lenormand et al. 1998).

Therefore, a reason for the fact that increased ERK1/2 activation following caveolin-1 siRNA transfection failed to cause promoted neurite outgrowth might be due to cytoplasmic sequestration and prevention of nuclear translocation of active ERK1/2. Indeed, PACAP-induced phosphorylation of ERK1/2 in nuclear fraction was significantly decreased in cells transfected with caveolin-1 siRNA compared to cells transfected with siRNA of random sequences or untreated cells (Fig 4.2.11.C upper panel), with un-affected total ERK1/2 levels (Fig 4.2.11.C middle upper panel). These results were further confirmed by the immunostaining with an anti-phospho-p42/44 ERK1/2 antibody (Fig 4.2.11.D). Clearly, in cells transfected with the control siRNA sequence, majority of the phosphorylated ERK1/2 induced by PACAP was located in the nuclear fraction (indicated by Hoechst staining), while in caveolin-1 siRNA-treated cells, the distribution of activated ERK1/2 was dispersed and the portion of phosphorylated ERK1/2 in the nuclei was much less than that in the control cells. Therefore, the nuclear translocation of PACAP-induced phosphorylated ERK1/2 was somehow inhibited by knock-down of caveolin-1 expression. Whether the decreased

amount of phosphorylated ERK1/2 allocated in nuclear fraction leads to decreased downstream transcription factor activation and subsequent attenuated neurite elongation will be examined in section 4.2.12.

C.

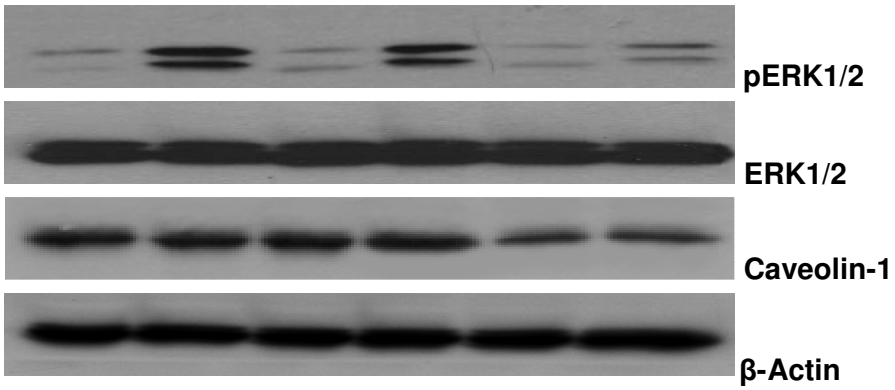


Fig 4.2.11. C. The effect of caveolin-1 siRNA on the pERK1/2 level in the nuclei of PACAP-stimulated PC12 cells. PC12 cells were treated with or without caveolin-1 siRNA or control siRNA before incubated with or without PACAP for 5 min. The cells were homogenized and the nuclear fractions of the cells were collected and subjected to Western blotting with pERK1/2 and ERK1/2 antibodies. The caveolin-1 expression level was evaluated 48 h of caveolin-1 transfection (β-actin as loading control). The bands are representative of three independent experiments.

D.

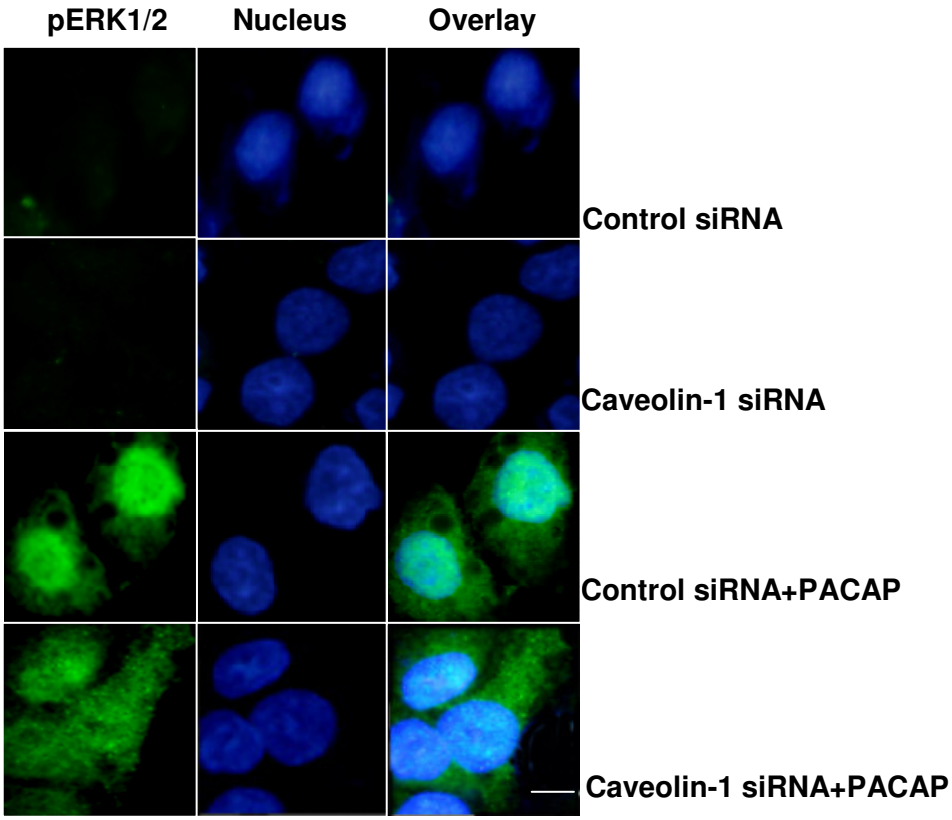


Fig 4.2.11. D. The effect of caveolin-1 on the nuclear translocation of PACAP-induced phosphorylated ERK1/2. PC12 cells were cultured on glass coverslips and were treated with or without caveolin-1 siRNA or control siRNA, respectively, followed by stimulation with PACAP (100 nM, 5 min), as indicated, before being fixed and stained with anti-phosphor-p42/44-ERK1/2 antibody. The cells were then cultured with goat-anti-rabbit IgG Alexa-488 secondary antibody (green) for 1 h, followed by staining with Hoechst33342 (blue) for 15 min at room temperature in the dark. The merged images were shown in right column. The micrographs shown are representative of three independent experiments. Bar length represents 10 μm for all micrographs.

4.2.12. ERK1/2-mediated CREB and Elk phosphorylation upon PACAP stimulation is an essential step for transcriptional regulation required for lipid rafts/caveolae-mediated PC12 cell differentiation

Many transcription factors, such as the phosphoprotein cyclic AMP response element binding protein (CREB) and ETS domain-containing protein (Elk), regulate gene expression in response to membrane depolarization, Ca^{2+} influx, cAMP-mediated second messenger systems, as well as MAPK activation. As mentioned previously, the nuclear translocation of ERK1/2 and phosphorylation of downstream transcription factors are essential for the activated ERK1/2 to affect different biological outcomes. Indeed, CREB is a major regulatory target of ERK1/2 signaling and may be pivotal for synaptic plasticity, neuronal survival and genesis of dendritic spines mediated by ERK1/2 (Montminy and Bilezikjian 1987; Impey et al., 1998 a, b; Bonni et al. 1999; Riccio et al. 1999; Waeber and Habener 1991; Lee 1997; Murphy and Segal 1997). Phosphorylation of CREB at Ser-133 is required for recruitment of the co-activator CREB-binding protein (CBP) and transcriptional activity (Gonzalez and Motminy 1989; Chrivia et al.1993). Numerous signaling events can activate CREB through phosphorylation of Ser-133, which contributes to many vital processes. Elk1, a member of the Ets family of transcription factors, is another nuclear target of ERK1/2 which is important for neuronal plasticity (Berman et al. 1998) and differentiation (Vossler et al. 1997). Elk is an important physiological substrate of ERKs and mediates serum-induced expression of immediate early genes (Marais et al. 1993).

The activation of ERKs can be assayed indirectly by measuring the transactivation of a 5× Gal4-E1b/luciferase reporter gene by an Elk-1/Gal4 chimera (Misra-Press et al. 1995). ERK-mediated Elk phosphorylation upon NGF stimulation is an essential step for transcriptional regulation required for neuronal differentiation (Vossler et al. 1997). Elk is phosphorylated by ERK at a cluster of Ser/Thr motifs at its C-terminus (Cruzalegui et al. 1999). Phosphorylation at these sites, particularly Ser-383, is critical for transcriptional activity of Elk1 (Marais et al. 1993; Zinck et al. 1993). However, evidence for CREB and Elk phosphorylation in response to lipid rafts/caveolae-mediated PACAP signaling is still lacking.

4.2.12.1. The involvement of transcription factors Elk and CREB in the PACAP-induced signaling pathways

In this study, the CREB and Elk activity was evaluated by Western blot using antibodies that recognize the phospho-Ser-133 of CREB, phospho-Ser-383 of Elk, CREB and Elk. As expected, PACAP stimulation increased both CREB (Fig 4.2.12.A) and Elk phosphorylation (Fig 4.2.12.B) compared to the unstimulated control cells. Blocking PKA activity by either H89 or PKI did not change the PACAP-induced CREB phosphorylation (Fig 4.2.12.A) or Elk phosphorylation (Fig 4.2.12.B). Since ERK1/2 phosphorylation levels were not influenced by PKA (Fig 4.2.6.B), it was not surprising that the downstream target transcription factors were not affected as well. However, blocking MEK1/2, cAMP, PLC and PKC by PD98059, RP-cAMP, U73122

and chelerythrine respectively, inhibited both the phosphorylation of CREB (Fig 4.2.12.A) and Elk (Fig 4.2.12.B). These results were also consistent with previous data showing that MEK1/2 (Fig 4.2.5.2.A), cAMP (Fig 4.2.4.B), PLC (Fig 4.2.9.B) and PKC (Fig 4.2.9.G) inhibitors reduced ERK1/2 activation. There was therefore a strong correlation between ERK1/2 activation and CREB, Elk-1 phosphorylation in PACAP signaling.

4.2.12.2. The role of lipid rafts/caveolae on Elk and CREB phosphorylation in PACAP-induced signaling pathways

Furthermore, when the plasma membrane was enriched with exogenous GM1 or cholesterol, the phosphorylation of both CREB (Fig 4.2.12.E) and Elk (Fig 4.2.12.F) were enhanced. On the other hand, when glycosphinoglipid or cholesterol levels were reduced by NB-DNJ or U18666a respectively, the phosphorylation of CREB (Fig 4.2.12.E) and Elk (Fig 4.2.12.F) were inhibited. Consistently, depletion of plasma membrane cholesterol by filipin, nystatin or methyl-β-cyclodextrin also attenuated the CREB (Fig 4.2.12.C) and Elk (Fig 4.2.12.D) activation. These results suggest that perturbation of lipid rafts/caveolae by modulating plasma membrane glycoshingolipid and cholesterol levels influenced the PACAP-stimulated phosphorylation of transcription factors CREB and Elk. Likewise, disrupting the caveolae/rafts by caveolin-1 siRNA (Fig 4.2.12.G) significantly inhibited PACAP-induced phosphorylation of CREB and Elk (Fig 4.2.12.G), with total CREB and Elk levels

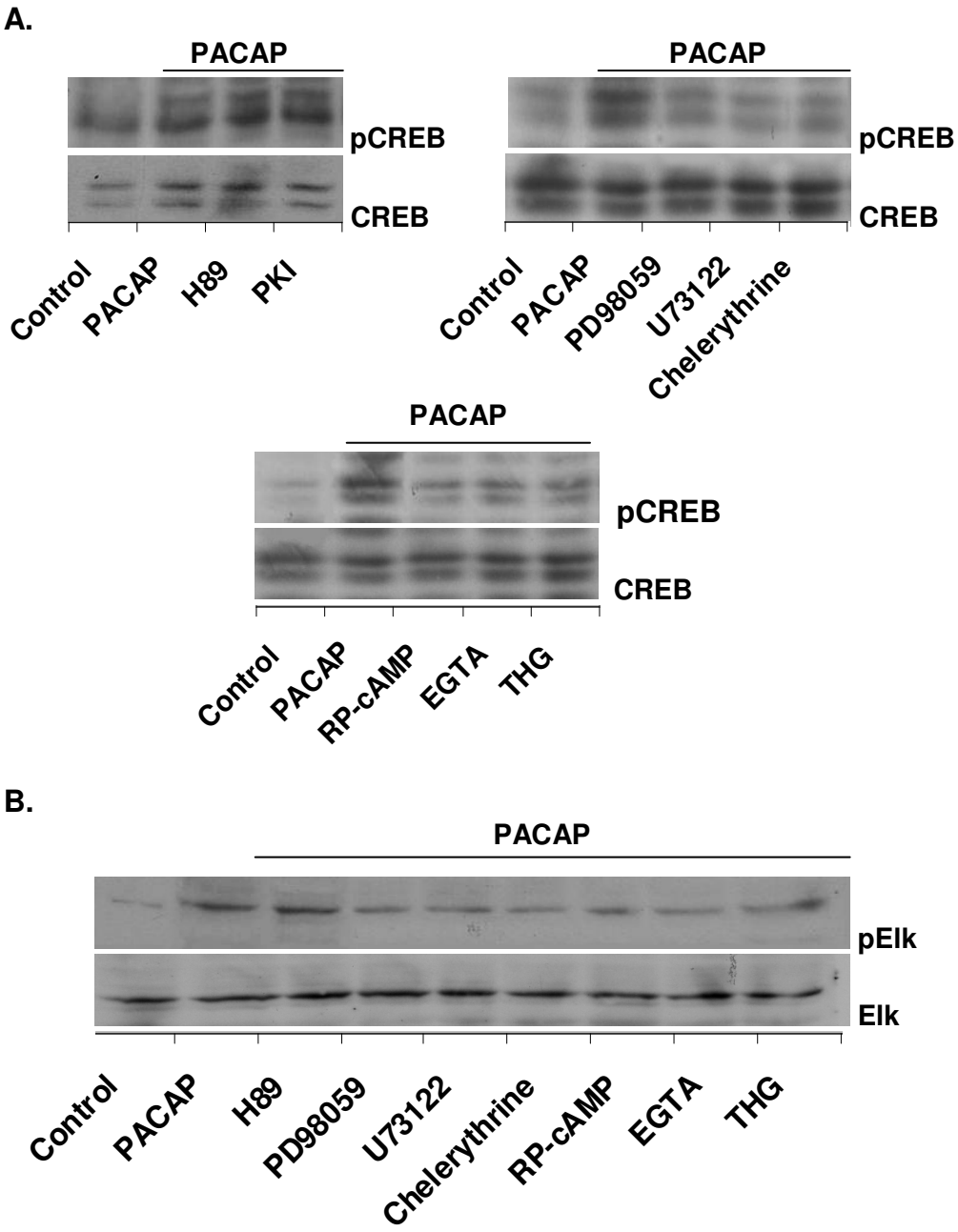
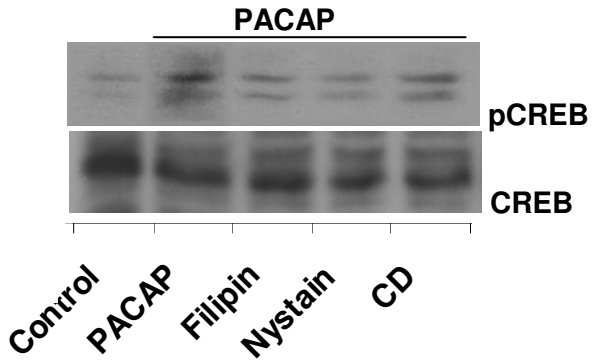


Fig 4.2.12. The involvement of transcription factors, CREB and Elk, in the PACAP-induced signaling pathways. PC12 cells treated with or without H89, PD98059, U73122, Chelerythrine, RP-cAMP, EGTA, THG for 1 h before brief incubation with or without PACAP for 5 min. The cells were harvested and the samples were subjected to Western blotting using a phosphospecific CREB (pCREB) **(A)** or a phosphospecific Elk (pElk) **(B)** antibody. Equal loading was verified by probing the blots with an anti-CREB (CREB) **(A)** or an anti-Elk (Elk) **(B)** antibody. The bands are representative of three independent experiments.

C.



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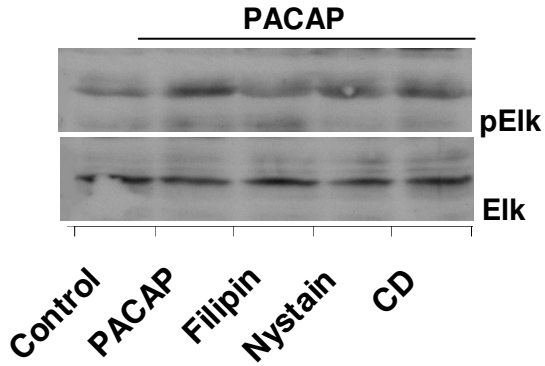

Fig 4.2.12. The effect of integrity of lipid rafts/caveolae on the phosphorylation of transcription factors, CREB and Elk, in PACAP-induced signaling pathways. PC12 cells were treated with or without filipin, nystatin, cyclodextrin (CD) for 1 h before brief incubation with or without PACAP (100 nM) for 5 min. The cells were harvested and the samples were subjected to Western blotting using a phosphospecific CREB (pCREB) **(C)** or a phosphospecific Elk (pElk) **(D)** antibody. Equal loading was verified by probing the blots with an anti-CREB (CREB) **(C)** or an anti-Elk (Elk) **(D)** antibody. The bands are representative of three independent experiments.

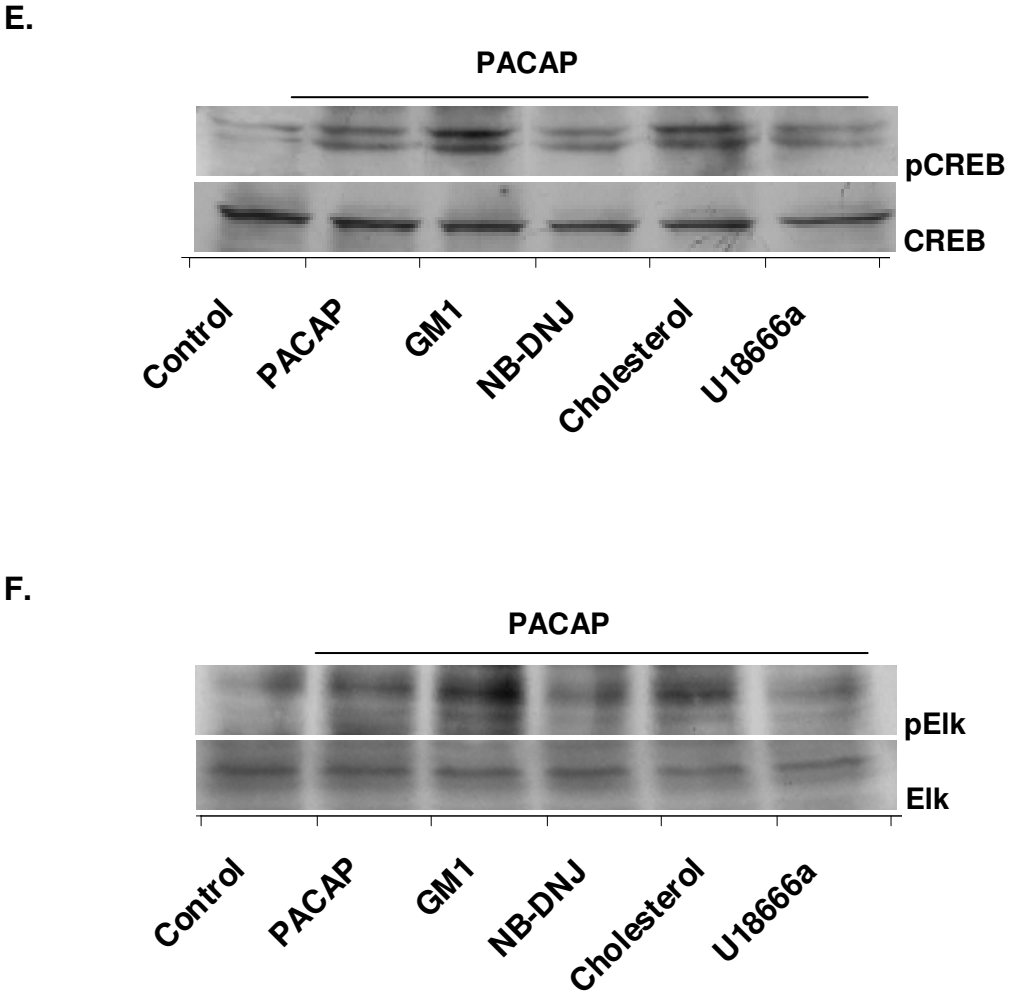

Fig 4.2.12. The effect of membrane glycosphingolipid and cholesterol levels on PACAP-induced CREB and Elk phosphorylation. PC12 cells were treated with or without GM1, NB-DNJ, Cholesterol-mβCD or U18666a for 24 h before brief incubation with or without PACAP for 5 min. The cells were harvested and the nuclear fractions of the samples were prepared as described in Materials and Methods. The samples were subjected to Western blotting using a phosphospecific CREB (pCREB) **(E)** or a phosphospecific Elk (pElk) **(F)** antibody. Equal loading was verified by probing the blots with an anti-CREB (CREB) **(E)** or an anti-Elk (Elk) **(F)** antibody. The bands are representative of three independent experiments.

unaffected. These data show that caveolin-1 siRNA attenuated downstream transcription factors activation, which could result from the inhibited nuclear translocation of phosphorylated ERK1/2 (Fig 4.2.11.C). Taken together, the above data confirm that perturbation of lipid rafts/caveolae affected PACAP-induced activation of Elk and CREB.

That concomitant phosphorylation of Elk and CREB coincides with the activation of cAMP, PLC, PKC, Ca^{2+} as well as downstream ERK1/2 suggests that the activation of both Elk and CREB were essential at the gene transcription level that were necessary for the neuritogenesis upon PACAP stimulation. This notion was further supported by another finding indicating that GSK3β, which was involved in PACAP signaling, is an inhibitory regulator of CREB. Inhibition of GSK3β blocks its inhibitory effect on the phosphorylation of CREB on Ser-133 (Grimes and Jope 2001). Therefore, Ser-9 phosphorylation of GSK3β, which attenuates the inhibitory effect of GSK3β, increases the phosphorylation of CREB. This conclusion was in agreement with previous finding in this study that increased GSK3β phosphorylation augmented ERK1/2 activation. This in turn, enhanced phosphorylation of downstream transcription factors.

G.

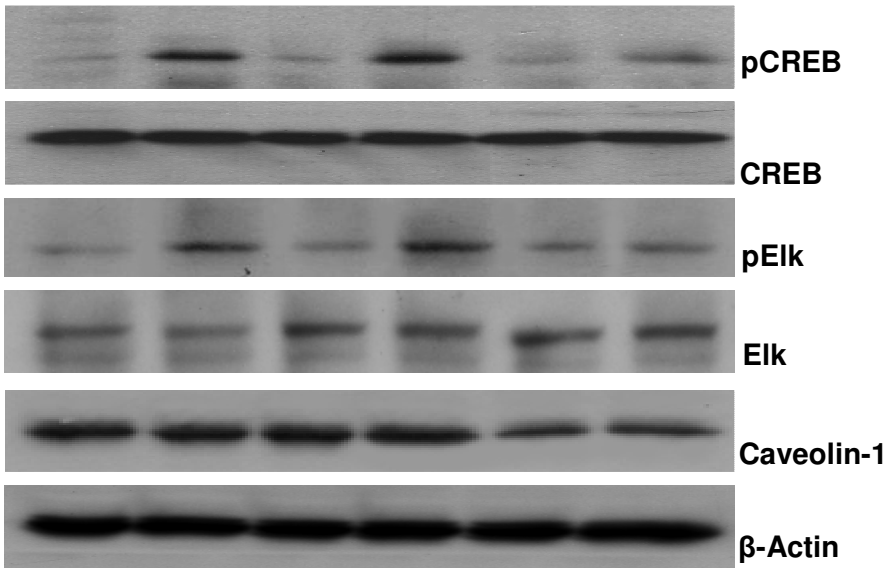

Fig 4.2.12. G. The effect of caveolin-1 siRNA on PACAP-induced CREB and Elk phosphorylation. PC12 cells were treated with caveolin-1 siRNA or control siRNA before incubated with or without PACAP for 5 min. The cells were homogenized and the nuclear fractions of the cells were collected and subjected to Western blotting with anti-pCREB and CREB antibodies or with anti-pElk and Elk antibodies. The caveolin-1 expression level was evaluated 48 h after caveolin-1 transfection (β-actin as loading control). The bands are representative of three independent experiments.

CHAPTER 5. DISCUSSION

5.1. PACAP-induced translocation of its receptor PAC1R into lipid rafts/caveolae, leading to enhanced cAMP generation and neurite outgrowth in PC12 cells

In PC12 cells, PACAP is known to activate the transmembrane adenylyl cyclase (AC) via its specific G-protein-coupled receptor PAC1R, resulting in an elevation of intracellular cAMP levels (Spengler et al. 1993). In the present study, I demonstrate that PACAP induces translocation of PAC1R into plasma membrane caveolae, where AC and the regulating G-proteins are localized (Schwencke et al. 1999; Ostrom et al. 2000a; 2000b; Insel et al. 2005). This colocalization of PAC1R and AC appears to enhance their interactions and contributes to the elevation of cAMP synthesis and eventually leads to neuritogenesis in PC12 cells. The results of this study elucidate for the first time the molecular events between PAC1R activation and cAMP production.

PC12 cells express PAC1R, and experience robust morphological changes following PACAP stimulation (Deutsch and Sun 1992; Ravni et al. 2006). Although PACAP has no significant effect on the expression of endogenous PAC1R mRNA (Cavallaro et al. 1995; Jamen et al. 2002), it slightly elevated cellular levels of PAC1R (Fig 4.2.1), implying that PACAP signaling might influence the rate of PAC1R translation

or its turnover. It is interesting to note that this PACAP-induced up-regulation of PAC1R was not affected by a disruption of the integrity of caveolin-enriched microdomains (Fig 4.2.1), which retarded PACAP-induced neurite outgrowth (Figs 3.2.1 and 3.2.2.A and C). This observation directly prompted us to examine the possible association of PAC1R with caveolae, where its effector proteins reside, following PACAP stimulation.

Immunoblot analysis revealed that PAC1R was localized in both detergent-soluble and -insoluble fractions of PC12 cells (Fig 4.2.2.B). Triton X-100-insoluble fractions were enriched with the caveolae-specific structural protein, caveolin-1 (Fig 4.2.2.A). Our data support a model in which a portion of PAC1R, originally localized in the detergent-soluble region, was recruited into caveolae after PACAP stimulation. Since AC, the effector of PACAP signaling pathway, accumulates in caveolae, this translocation of PAC1R was expected to enhance its coupling with AC. This was indeed demonstrated by the increased degree of co-immunoprecipitation of PAC1R and AC (Fig 4.2.2.D). As a result of this increased interaction, the intracellular cAMP level was markedly elevated (Fig 4.2.4.A) and consequently the signaling pathway leading to neurite outgrowth of PC12 cells was activated. Exactly how PAC1R translocated into caveolae upon PACAP stimulation remains unclear in terms of biophysical details. Nevertheless, translocations of activated receptors into lipid rafts/caveolae as means to initiate signaling cascades were reported before (Higuchi et al. 2003; Chini and Parenti 2004; Insel et al. 2005).

Caveolae are tiny (50 - 100 nm in diameter) plasma membrane invaginations enriched in cholesterol and glycosphingolipids. Nystatin and filipin disrupt caveolae by binding to cholesterol (Rothberg et al., 1992; Schnitzer et al., 1994; Orlandi and Fishman, 1998). These compounds blocked the redistribution of PAC1R (Fig 4.2.2.B) and retarded the neurite outgrowth of PC12 cells (Fig 3.2.2.A), suggesting that intact caveolae were necessary for the propagation of the PACAP signal. Clearly, activation of AC, the immediate downstream effector of PAC1R, was inhibited by treatments with these cholesterol-binding drugs, as demonstrated by the decrease in PACAP-induced cAMP synthesis (Fig 4.2.4.A). The importance of caveolar integrity in PACAP signaling was further investigated through another set of complementary experiments, focusing on glycosphingolipids, another major lipid constituent of caveolae. The cellular glycosphingolipid composition can be modulated by administration of exogenous gangliosides (Farooqui et al. 1997; Simons et al. 1999; Crespo et al. 2002; Liu et al. 2004), transfection of cells with a glycosphingolipid synthase gene (Mitsuda et al. 2002; Nishio et al. 2004) or use of inhibitors of glycosphingolipid biosynthesis (Platt et al. 1994a; 1994b; Hynds et al. 2002; Nagafuku et al. 2003). The former two approaches are expected to increase glycosphingolipid level at the plasma membrane surface while the third will reduce it. In this study, the ganglioside GM1 level was modulated by exposure of the cells to exogenous GM1 or treatment with NB-DNJ, a glucosylceramide synthase inhibitor. Although quantitative roles of gangliosides in membrane microdomians such as

caveolae were unclear, it was believed that the carbohydrate-containing sphingolipids would affect the integrity and stability of these structures such that signaling proteins were excluded from or recruited into them (Simons et al. 1999; Mitsuda et al. 2002; Liu et al. 2004; Sottocornola et al. 2006). Apparently, increasing membrane gangliosides by addition of exogenous GM1 promoted translocation of PAC1R into the caveolin-1-enriched detergent-insoluble fractions, while reduction of membrane gangliosides inhibited this process (Fig 4.2.2.B). Although direct interactions between gangliosides and membrane proteins were reported before (Mutoh et al. 1995; Miljan et al. 2002), it is unknown at this stage whether the presence of more PAC1R in the detergent-insoluble fraction was due to GM1-induced stabilization and/or enlargement of the existing caveolae which could therefore accommodate more PAC1R molecules, or to direct interactions between GM1 and PAC1R which might help to retain more PAC1R in the caveolae.

In agreement with the pharmacological evidence above, down-regulation of caveolin-1 expression by siRNA confirmed that intact caveolae were necessary for PAC1R translocation and downstream PACAP signaling via cAMP. As a consequence of caveolin-1 knockdown, neurite outgrowth of PC12 cells was inhibited (Fig 3.2.2.C), PAC1R translocation was blocked (Fig 4.2.2.B) and PACAP-induced cAMP generation was diminished (Fig 4.2.4.A). *In vivo* experiments with caveolin-1-null mice showed a remarkable lack of caveolae in various tissues (Drab et al. 2001; Razani et al. 2001; Le Lay and Kurzchalia 2005), implying that caveolin-1 is

necessary for the formation of caveolae in cells. Knockdown of caveolin-1 would therefore be expected to disrupt the structure of caveolae which might cause their ablation (Hong et al. 2004). Taken together, these results provided compelling evidence that caveolae played a pivotal role in mediating PACAP signaling in PC12 cells.

Another important result in support of PACAP-induced caveolar translocation of PAC1R was the observation of patchy plasma membrane distribution of the receptors following PACAP stimulation, while those in untreated controls showed a rather uniformed distribution (Fig 4.2.3). Fluorescence staining of PAC1R and GM1, the marker lipid for caveolae, demonstrated their increased co-localization in the membranes of stimulated cells. This was in agreement with the observation by immunoprecipitation (Fig 4.2.2.B), where PACAP induced translocation of PAC1R into detergent-insoluble fractions. I also investigated the effects of glycosphingolipid concentration on the distribution pattern of PAC1R (Fig 4.2.3). As discussed above, the concentration of ganaglioside GM1 in the plasma membrane can be conveniently modulated and visualized. Clearly, PACAP-induced distribution of PAC1R into membrane patches was retained at increased GM1 concentrations, while treatment with NB-DNJ, which significantly decreased cellular ganglioside levels, resulted in dispersion of the patched PACAP receptors. These observations imply that the integrity of glycosphingolipid-enriched microdomains was essential for retaining PAC1R in these structures.

AC6 (type 6 adenylyl cyclase, a calcium-inhibitable isoform) is the major adenylyl cyclase in PC12 cells and is co-localized in cholesterol- and sphingolipid-enriched microdomains (Oshikawa et al. 2003) with G-proteins (Schwencke et al. 1999; Ostrom et al. 2000b; Insel et al. 2005) and caveolin-1 (Peiro et al. 2000 and this study). Therefore, translocation of PAC1R into these microdomains serves as a determinant for effective coupling of the receptor to its effector molecule, AC6. Indeed, enhanced AC6 co-immunoprecipitation using anti-PAC1R antibody was observed following PACAP stimulation (Fig 4.2.2.D). The increased coupling efficacy appeared to contribute to the increased ability of PAC1R in stimulating AC6, leading to an elevation of intracellular cAMP levels (Fig 4.2.4.A). These data provide direct evidence for the functional significance of co-localization of PACAP signaling molecules in caveolin-enriched membrane microdomains.

In conclusion, the current study on PACAP signaling transduction in PC12 cells demonstrates that, following PACAP stimulation, translocation of PAC1R into caveolae enhanced the efficacy of PAC1R-AC coupling, which in turn increased intracellular cAMP generation, leading to the activation of the downstream pathways for neurite outgrowth. On the other hand, it could be speculated that, in neurons lacking caveolae, similar mechanism might exist for caveolin-enriched microdomains and it is definitely worth further investigation.

5.2 Lipid rafts/caveolae-mediated PACAP signaling cascades and downstream events

5.2.1. Modulation of the ERK pathway by EPAC and GTP-loaded Rap1 involving the concomitant activation of Ras, PKC and Ca^{2+}, resulting in nuclear transcription in PACAP signaling in PC12 cells

Our present data indicate that PACAP activated the ERK pathway through the pleiotropic stimulation of several signaling pathways acting synergistically, leading to the overall effect of neurite outgrowth of PC12 cells. Cyclic AMP, upon activated by Gs-coupled GPCR-PACAP receptor type 1, amplifies the signaling by acting on its effector proteins. Formerly, PKA was thought to be the major effector for cAMP. However, in our system, PACAP-induced signaling was apparently independently of PKA (Fig 4.2.6). This observation was in line with similar findings by others (Lazarovici et al. 1998). The molecular events leading from PACAP-induced cAMP elevation to ERK1/2 activation were therefore lacking. Instead, we observed that activation of EPAC, a Rap1 guanine nucleotide exchange factor that mediates Rap1 activation by direct binding to cAMP, potentiated PACAP-stimulated ERK1/2 activation and neurite elongation (Figs 4.2.7.B and C). These data were consistent with previous findings that EPAC agonist attenuated the inhibitory effect of AC inhibitors on the GPCR-stimulated ERK activation (Lin et al. 2003). EPAC was

therefore likely to be involved in PACAP signaling. Furthermore, I found that PACAP stimulated GTP-loading of both Rap1 and Ras (Figs 4.2.8.1.A and 4.2.8.3.A). The stimulation on Rap1 was more pronounced and sustained than that on Ras, suggesting that Ras might primarily be involved in a transient stimulation of ERK while Rap1 was likely responsible for the prolonged activation of ERK and its subsequent nuclear translocation, which is known to be necessary for cell differentiation (Marshall 1996). However, how these GTP-binding proteins were activated upon PACAP stimulation, particularly, whether Rap1 activation was directly modulated by EPAC or whether Rap1 could be activated by other signaling intermediates are matters of interest for further investigations.

Our findings suggest that Rap1 activation requires the proper function of lipid rafts/caveolae. Depletion of membrane rafts glycosphingolipid or cholesterol by the rafts disruptors reduced Rap1 activation, whilst enrichment with GM1 or cholesterol increased the GTP-loading of Rap1 (Fig 4.2.8.2.A). In addition, the distribution pattern of membrane-associated Rap1 at the plasma membrane changed upon PACAP stimulation and perturbation of rafts/caveolae (Fig 4.2.8.2.B). It was translocated to the caveolae fraction of plasma membrane upon induction of PACAP, where more receptors and signaling molecules are present. GM1, which stabilizes and maintains caveolae, enhanced this shift. On the other hand, caveolar disruptors dispersed Rap1 to non-caveolae fractions. These observations suggest that lipid rafts/caveolae act as a biophysical hub for PACAP signaling, from the upstream receptor (PAC1R)

translocation and coupling of AC, to downstream signaling intermediates such as Rap1.

Although PACAP-elicited cAMP elevation and subsequent EPAC activation were shown to be essential for PACAP-induced ERK1/2 stimulation (Fig 4.2.8.1), EPAC activation itself was not sufficient to induce neurite extension and efficient ERK1/2 stimulation (Figs 4.2.7 A and C). The proximal effectors of PAC1R are AC and PLC, whose activations resulted in the stimulation of a set of kinases, directly controlled by second messengers, *ie.* cAMP, PKC and Ca^{2+}. Indeed, I found that cAMP and PLC were both involved in some way. However, PAC1R might exert its major function through cAMP elevation rather than PLC or PKC activation. This hypothesis was supported by the fact that different extent of neurite outgrowth was induced by the cAMP analog db-cAMP, compared to that by the PKC activator PMA. cAMP on its own induced neurite elongation robustly (Fig 4.2.4.C), while PMA alone could not elicit the formation of long neurites (Fig 4.2.9). Nevertheless, PMA significantly enhanced PACAP-elicited neurite outgrowth. In addition, inhibition of PKC activity also attenuated neurite elongation (Fig 4.2.9.E) and ERK phosphorylation (Fig 4.2.9.H) induced by PACAP in PC12 cells. These findings were in agreement with the results obtained by others (Bouschet et al. 2003), suggesting that PKC was involved in the activation of ERK1/2 by PACAP. PKC activation may therefore act to enhance cAMP signaling upon PACAP binding to PAC1R, and the cAMP and PKC pathways were likely to cooperate together in PACAP signaling.

It is known that activation of PLC results in the hydrolysis of phosphatidylinositol biphosphate (PIP_2) and generation of IP_3 and diacylglycerol, which activates PKC. IP_3, in turn, stimulates the release of Ca^{2+} from the smooth ER and activates PKC through diacylglycerol. Since PLC and PKC were both activated upon PACAP binding to PAC1R in PC12 cells, whether the intracellular Ca^{2+} mobilization also affects the PACAP signaling is worth checking. Indeed, treatment of the cells with either the extracellular Ca^{2+} chelator EGTA or the intracellular Ca^{2+} release promoter THG (Figs 4.2.9.E, F, G and 4.2.10.E) inhibited PACAP-induced neurite outgrowth and ERK1/2 phosphorylation. These results suggest that Ca^{2+} mobilization played a role in PACAP signaling in PC12 cells. Our data indicate that stimulation of PACAP signaling might elicit the concomitant activation of PLC, PKC and Ca^{2+} pathways, which engaged in collaboration with cAMP to potentiate the effect of GTP-loaded Rap1. For full neurite extension, all of these intermediates might exert their functions on ERK1/2 phosphorylation by regulating the activity of another signaling intermediate, GSK3β (Figs 4.2.10.G, H, I, J).

GSK3β is an enzyme first characterized by its ability to phosphorylate and inhibit glycogen synthase (Embi et al. 1980; Rylatt et al.1980). It is now known to be a key component of several intracellular signaling pathways (Plyte et al. 1992; Grimes and Jope 2001; Kim and Kimmel 2000). The best characterized function of GSK3β is in the survival-promoting signaling pathways involving PI3K and Akt (Datta et al. 1999)

downstream of epidermal growth factor (EGF) and insulin-like growth factor (IGF) signaling. However, information on possible functions of GSK3β in neuronal differentiation is lacking. In the current study, I demonstrate a novel role for GSK3β in both PACAP-induced ERK1/2 activation (Fig 4.2.10.I) and neurite outgrowth (Figs 4.2.10.A, B, C, D) in PC12 cells. GSK3β appeared to be responsible for linking PACAP-elicited signaling to the downstream ERK1/2 activation. It is known that GSK3β also functions in regulation of transcription factors activation (Grimes and Jope 2001) and modulation of cytoskeleton stability (Hanger et al.1992; wagner et al. 1996; Trivedi et al. 2005; Goold and Gordon-Weeks 2004; Yoshimura et al.2005). GSK3β is therefore conceivable as a signal transducer that is responsible for the effective neurite outgrowth induced by PACAP in PC12 cells. Consistent with this notion, GSK3β activity was regulated by Rap1 (Fig 4.2.10. H), PKC and Ca^{2+} (Fig 4.2.10.G) in PACAP signaling. Moreover, intact caveolae microdomains were required for the proper function of GSK3β on the regulation of ERK1/2 activation (Figs 4.2.10.E and F). These data suggest a mechanism to control Rap1 activity by GPCRs and their downstream intermediates.

Finally, the nuclear translocation of ERK1/2 and activation of downstream transcription factors are known to be essential for the neuronal plasticity and survival (Bonni et al. 1999; Riccio et al. 1999). Both the transcription factors, Elk and CREB, are important physiological substrates of ERKs and mediate immediate early genes (Marais et al. 1993). In NGF signaling, Elk phosphorylation is critical for neuronal

differentiation (Vossler et al. 1997). However, evidence of downstream transcription factor activation upon ERK1/2 phosphorylation stimulated by PACAP has been lacking. In this study, I demonstrate that PACAP-induced ERK1/2 activation participated in the regulation of both CREB- and Elk-dependent transcription (Figs 4.2.12.A and B). The activation of these transcription factors required not only the necessary intermediates in the pathways (Figs 4.2.12.A and B), but also intact plasma membrane rafts/caveolae microdomains (Figs 4.2.12.C, D, E, F, G). Indeed, disruption of caveolae by caveolin-1 siRNA inhibited ERK1/2 nuclear translocation (Figs 4.2.11.C and D) and subsequent activation of CREB and Elk (Fig 4.2.12.G), although the cytoplasmic ERK1/2 phosphorylation was increased (Figs 4.2.11.A and B). As a result, PACAP-stimulated neurite elongation was eventually inhibited by caveolin-1 siRNA (Fig 3.2.5.A). However, reasons for the impairment of ERK1/2 nuclear translocation after perturbation of caveolae by caveolin-1 siRNA are still unknown at this stage. Whether it was related to the malfunction of the endocytic function of membrane caveolae or the architecturally impaired nuclear membrane due to caveolin-1 knockdown, or resulted from other underlying mechanisms are of interest for further investigations.

I have observed that cholesterol-chelators filipin, nystatin or methyl-β-cyclodextrin also increased PACAP-induced ERK1/2 activation (data not shown). One possible reason for these effects was that depletion of plasma membrane cholesterol results in ligand-independent activation of epidermal growth factor receptor (EGFR) and

subsequent activation of ERK1/2 by PI3K and Ras (Chen and Resh 2002). Accumulating evidence suggests that the primary function of EGFR is in the regulation of cell proliferation (Barnard et al. 1995; Khasharyasha et al. 1993; Kanae et al. 2005). Therefore, the enhanced ERK1/2 phosphorylation by cholesterol-depletion might not be due to the induction of PACAP, but rather to the increased activation of EGFR. Whether the proliferation was indeed increased after cholesterol-removing in PACAP-induced PC12 cells is of interest for further confirmation. If this is the case, this fraction of activated ERK1/2 would contribute to proliferation instead of differentiation. The elevated ERK1/2 phosphorylation is therefore not necessarily led to enhanced neurite extension. Rather, the specific receptor(s) and the signaling cascade(s) that stimulate ERK1/2 activation have a decisive influence on the outcome of the biological response.

5.2.2. The inhibitory effect of cAMP on Ras activation in PACAP signaling

One interesting finding obtained in this study is that RP-cAMP, an antagonist of cAMP, increased PACAP-elicited Ras GTP-loading (data not shown). The overall level of Ras activation was augmented after the inhibition of cAMP activity, suggesting that Ras activation was in fact counteracted by cAMP-EPAC-Rap1 signaling in PACAP stimulation. Indeed, whether cAMP inhibits or enhances ERK activation is cell type dependent (Dumaz and Marais 2005). In PC12 cells, cAMP are thought to stimulate ERK in a B-Raf-dependent manner (Jaiswal et al. 1994).

However, the mechanisms by which ERK can be modulated by Raf protein and the crosstalk between cAMP-Rap1 and Ras are still unclear. One of the reported functions of Rap1 is its interaction with, and modulation of the Ser/ Thr kinases Raf1 and B-Raf. This occurs through an interaction with both the Raf binding domain (RBD) and the adjacent cysteine-rich region (CRD) of Rap1 (Herrmann et al. 1996; Okada et al. 1999), resulting in the modulation of ERK and ERK-mediated transcriptions. Furthermore, Rap1 possesses an effector domain that is similar to that of Ras, indicating that both small GTPases might share common downstream effectors (Bos et al. 2001). Therefore, Rap1 may antagonize Ras-dependent ERK1/2 signaling through competitive interaction with the RBD of B-Raf (a neuron-specific Raf isoform which is highly expressed in PC12 cells), and preventing it from activating Ras. Nevertheless, B-Raf has been suggested to block Raf-1 activation by Ras when over-expressed (Okada et al. 1999). Another way how Rap1 might function to modulate Ras/ERK signaling is that Rap1 binds tightly to Raf-1 CRD, preventing it from interacting with Ras. Thus when both Ras and Rap1 interact with the RBD and the CRD respectively, the Raf activation complex might be locked in a refractory state (Hu et al. 1997). When Raf-1 CRD is replaced by the lower-affinity B-Raf RBD, Rap1 is converted into an activator. However, whether Rap1 modulate Ras/ERK signaling by trapping Raf isoforms in an inactive complex, and how the Raf-1 CRD is replaced by B-Raf RBD, requires further investigations. A previously uncharacterized feedback loop from ERK to Raf has also been proposed (Alessi et al. 1995). Raf is activated by phosphorylation, which occurs when it is recruited to the

plasma membrane by Ras, suggesting a possible feedback regulation mediated by Ras in cAMP-Rap1 signaling.

5.2.3. The essential role of cytoskeleton (actin and microtubule) stability for the neuritogenesis process induced by PACAP in PC12 cells

It is known that a critical aspect of neuronal differentiation is the rearrangement of the neuronal cytoskeleton, leading to specific and directed elongation of microtubules into growing neuritic cytoplasmic extensions. Therefore, the involvement of GSK3β, which regulated ERK1/2 activation and several cytoskeleton-associated molecules in the neuronal differentiation process, further illustrates the essential role of cytoskeleton played in PACAP-induced neurite elongation of PC12 cells. Since cytoskeletons are major and required constituent of neurites, and ERK1/2 may regulate the assembly and stability of neuritic cytoskeleton elements, the present observations that GSK3β was involved in PACAP-induced neurite outgrowth and ERK1/2 phosphorylation suggest a possible causal relationship between these two events. Moreover, sustained ERK activation leads to translocation of ERKs to the nucleus (Traverse et al. 1992; 1994; Nguyen et al.1993; Dikic et al. 1994) and subsequently activation of downstream transcription factors CREB and Elk (Fig 4.2.13). The latter would result in the modulation of relevant gene transcription and subsequent protein synthesis to supply raw materials for neurite elongation.

The dynamic filamentous cytoskeleton actin network might also be linked to lipid rafts/caveolae and certain membrane-bound molecules. It has been found that perturbation the stability of the actin filament disrupted PACAP-elicited neurite elongation (data not shown). Demolishing the integrity of lipid rafts/caveolae by either cholesterol deprivation or caveolin-1 depletion, in turn, lead to the dysfunction of rafts-associated proteins and disruption of the cortical actin ring. Accordingly, it is likely that the detrimental effect of rafts/caveolae disruption might, at least in part, arise from an impairment of the signaling pathways associated with cytoskeleton stability. Therefore, it is comprehensible that without a functional cytoskeleton, neurite extension would be fundamentally retarded.

5.3. Future directions

The current study on PACAP signaling transduction in PC12 cells demonstrated that the neurotrophic factor PACAP binding to its specific receptor PAC1R enhanced its membrane association with lipid rafts/caveolae and subsequently facilitated the receptor and effector protein interaction. This coupling of PAC1-AC positively contributed to intracellular cAMP production, which subsequently regulated downstream signaling molecules ERK1/2 activation through Rap1 and GSK3β. Rap1, as a membrane associated protein, targeted to lipid rafts/caveolae upon activation and may therefore facilitate the signaling transduction.

On the other hand, Ras activity was also found to be involved in the PACAP-stimulated neurite extension process. Several isoforms of Ras have been identified, such as H-Ras, K-Ras and N-Ras, and have multiple effector proteins (Raf-1, A-Raf, B-Raf, PI3K and Ral-GEF) (Sun et al. 2006). Targeting of specific Ras isoforms to distinct microdomains within the plasma membrane contribute to the different signaling capacities of Ras isoforms (Yan et al. 1998). The identification of specific isoform(s) of Ras that has (have) been activated upon PACAP stimulation and the mechanisms of how the Ras isoform(s) is (are) activated in this signaling, therefore, are worth further investigation. Particularly, the distribution pattern of Ras in different PM microdomains upon perturbation of the stability of lipid rafts/caveolae in PACAP signaling would be important to document. Conventionally, Ras activation was mediated by receptor tyrosine kinase (RTK). Multiple pathways have recently been reported that could indirectly stimulate Ras-dependent ERK activation. For example, GPCR-mediated Ras activation through Gβγ of G protein, which subsequently recruits c-Src/PI3K, phosphorylates SHC and finally leads to Grb2/Sos1 recruitment and Ras activation (Marinissen and Gutkind 2001). Gqα of G protein could also indirectly activate Ras via Gqα-PLCβ pathway (Lev et al 1995). In addition, Ras could also be activated by cAMP through direct binding to cyclic nucleotide Ras GEF (cNrasGEF) in response to elevated intracellular cAMP in a PKA-independent manner (Pham et al 2000). Therefore, it is worthwhile to investigate further the upstream events that activate Ras in PACAP signaling, which may lead to better understanding the synergistical action of Rap1 and Ras in these pathways.

In addition, EPAC was found to enhance the PACAP signaling in PC12 cells. However, it could not induce neurite outgrowth on its own (Fig 4.2.7), and the mechanisms of its function is not yet clear. It has been shown that EPAC could not activate ERK unless it was recruited to the plasma membrane (Wang et al 2006). Whether EPAC activates a pool of Rap1 that could induce downstream ERK activation through targeting to the plasma membrane, particularly lipid rafts, upon PACAP stimulation, is another interesting issue to be addressed.

The current study on PACAP-induced differentiation transduction pathways in PC12 cells shows that lipid rafts/ caveolae play essential role in the signaling cascade, from the upstream receptor translocation, coupling of effector protein AC to the downstream signaling molecules activation and targeting, such as Rap1. Whether the similar mechanisms are utilized to regulate neuritogenesis in primary neurons needs further investigation.

CONCLUSION

Lipid rafts/caveolae microdomains differ from the rest of plasma membrane in being specifically enriched in glycosphingolipids, cholesterol and a myriad of intracellular signaling proteins (Simons and Ikonen 1997). These microdomains form relatively stable lipid matrix which act as an ordered support for receptor mediated signaling events. In this study, we aim to characterize the role of lipid rafts/caveolae in the neuritogenesis process induced by neuropeptide PACAP in PC12 cells, as well as the signaling pathways involved in this process.

In the first part of this study, the effects of the major components of lipid rafts/caveolae: cholesterol and glycosphingolipid and the integrity of lipid rafts/caveolae on PACAP-induced neuritogenesis were examined in PC12 cells. These effects were implemented by either inhibition of *de novo* biosynthesis of glycosphingolipids, inhibition of cholesterol intracellular trafficking or depletion of cholesterol level on PC12 cells in the presence of PACAP. Treatment of the cells by the above inhibitory factors hindered neurite outgrowth, while supplementation of the cell plasma membrane with exogenous ganglioside GM1 or cholesterol restored neurite outgrowth. These data suggest that removing either cholesterol or glycosphingolipids of the lipid rafts/caveolae inhibits neurite outgrowth, and this inhibition is reversible once the cells are replenished with the depleted components. These data also indicate that the two major components of the lipid rafts/caveolae

microdomains: glycosphingolipid and cholesterol were essential in promoting neuritogenesis. However, perturbation of the integrity of lipid rafts/caveolae by silencing the caveolar structural protein caveolin-1, the inhibitory effects on the PACAP-induced neurite outgrowth was irreversible, suggesting that the proper function of lipid rafts/caveolae was essential for PACAP-mediated neuritogenesis.

To unveil the underlying mechanisms of lipid rafts/caveolae's role in PC12 neuritogenesis, the level of PACAP receptor type I (PAC1R) and the ratio of the PAC1R level in lipid rafts to non-raft fractions of the plasma membrane after the treatment of the cells with different caveolae disruptors were examined. It was found that PAC1R shifts to the caveolin-enriched Triton X-100-insoluble microdomains after PACAP induction. GM1, which promoted neurite extension, enhanced this shift. Perturbations of lipid rafts/caveolae, which retarded neurite outgrowth, prevented the translocation of PAC1R into the detergent-insoluble membrane microdomains. In addition, the altered membrane distribution of PAC1R facilitated its interaction with AC, the target enzyme located in caveolae and catalyzes the conversion of ATP to cAMP. Nevertheless, perturbation of caveolae did not significantly affect expression of PAC1R in PC12 cells. These results suggest that it was the PAC1R's shift to the lipid rafts/caveolae fraction that results in the downstream signaling and eventual neurite extension.

Based on the above results, further elucidations of the signaling pathway(s) downstream of the PAC1R and the effect of the lipid rafs/caveolae on the PACAP signaling would be important. Indeed, our results show that the translocation of PAC1R into caveolae lead to generation of cAMP and activation of PLC. However, instead of PKA, EPAC and Rap1 were activated upon cAMP elevation. These activations were favored the detergent-insoluble fraction upon PACAP induction, providing additional evidence for the importance of lipid rafts/caveolae in PACAP-induced neurite differentiation of PC12 cells. Known as a plasma membrane-associated protein, Rap1's activation was correlated with the integrity of lipid rafts/caveolae. Rap1 regulated PACAP-induced neurite outgrowth through modulating GSK3β activity and subsequent MEK1/2-dependent ERK1/2 phosphorylation. On the other hand, PKC and Ca^{2+} mobilization collaborated with the cAMP pathway of PACAP signaling by regulating GSK3β and ERK1/2 activity. In addition, PACAP-elicited Rap1 and Ras activation accounted for both the sustained and transient activation of downstream ERK1/2 phosphorylation and neurite extension. Activated ERK1/2 translocated to the nucleus and phosphorylated the transcription factors Elk and CREB. This was an essential step of transcriptional regulation required for rafts/caveolae-mediated PC12 cell differentiation. The results revealed novel components of PACAP-induced signaling cascades in PC12 cells that were mediated by lipid rafts/caveolae, downstream of PAC1R (Fig 5.1).

The present study shows that lipid rafts/caveolae microdomains act as a hub for the molecules involved in the PACAP-induced differentiating PC12 cells to gather upon receptor-ligand binding. Specifically, it demonstrates that the PAC1R receptor shifts to the rafts/caveolae once activated, and initiates downstream signaling. These findings are of importance since they show for the first time a general picture of the neuritogenesis process and underlying mechanisms in differentiating PC12 cells induced by PACAP as well as its signaling pathways. They also indicate that one key factor for neuritogenesis is the integrity of the lipid rafts/caveolae. However, further work is needed to confirm that these observations in PC12 cells are generally applicable to other cell lines, especially primary neurons. With a further understanding of the mechanisms of neuritogensis in general, the mechanisms underlying of neurodegeneration and neuronal regeneration after injury could be further comprehended, which may lead to better prevention and therapy.

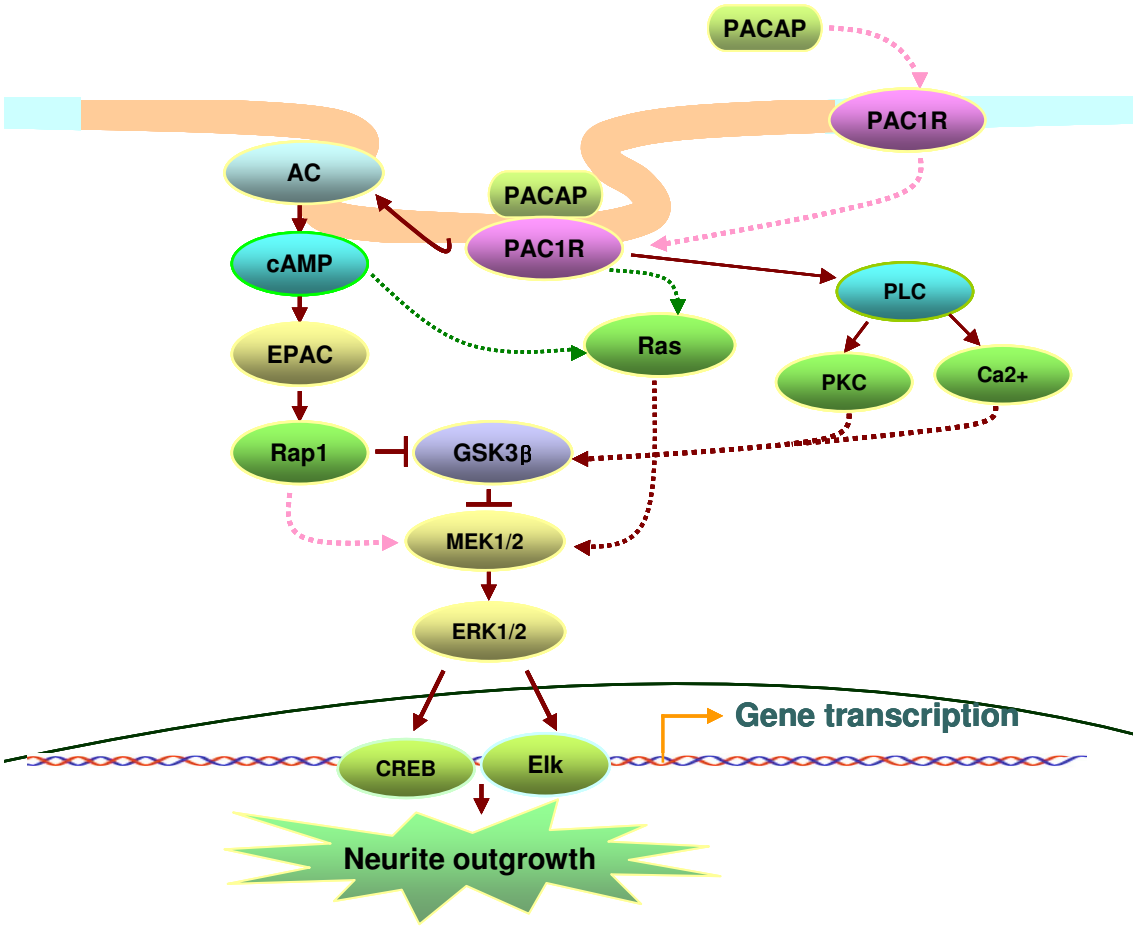

Fig 5.1. A schema of the signaling transduction pathways of PACAP-induced differentiation in PC12 cells.

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APPENDIX

Media and buffers used in this study

1. Reagents for cell culture

Complete RPMI 1640 culture medium

RPMI 1640 medium supplemented with 10% (v/v) horse serum and 5% (v/v) heat-inactivated fetal bovine serum.

Cell freezing medium

RPMI 1640 medium supplemented with 40% (v/v) heat-inactivated fetal bovine serum and 10% (v/v) dimethly sulfoxide (DMSO).

Lysis buffer

50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, protease inhibitor cocktail.

PBS buffer

1.76 mM KH_2PO_4, 10 mM Na_2HPO_4, 137 mM NaCl, 9 mM KCl, pH 7.4

Trypsin-EDTA

0.05% trypsin and 0.02% EDTA

2. Reagents for SDS-PAGE and Western blotting

4 × Resolving gel buffer

1.5 M Tris-HCl, pH 8.8, SDS 4%

4 × Stacking gel buffer

0.5 M Tris-HCl, pH 6.8, SDS 4%

5 × Loading buffer

10% SDS, 50% sucrose, 0.1% electrophoresis purity reagent bromophenol blue, 1 M Tris-HCl (pH 6.8) and 10% (v/v) β-mercaptoethanol

10 × Transfer buffer

3.0% Tris and 14.4% glycine

1 × Transfer buffer

10% (v/v) 10 × Transfer buffer supplemented with 70% (v/v) distilled water and 20% (v/v) methanol

5 × Running buffer

1.5% Tris-HCl, 7.2% glycine and 0.5% SDS

5 × TBS

6.1% Tris and 8.8% NaCl, pH 7.5

1 × TBST

0.1% TWEEN 20 in 1×TBS buffer

Blocking buffer

5% skim milk in TBST

3. Reagents for sucrose density gradient centrifugation

Homogenisation buffer (HB)

250 mM sucrose, 3 mM imidazole, 1 mM EDTA and protease inhibitor cocktail (1 tablet /10 ml HB), pH 7.4

10% Sucrose (in HB)

10% sucrose, 3 mM imidazole, 1 mM EDTA and protease inhibitor cocktail (1 tablet / 10 ml HB), pH 7.4

40% Sucrose (in HB)

40 % sucrose, 3 mM imidazole, 1 mM EDTA and protease inhibitor cocktail (1 tablet / 10 ml HB), pH 7.4